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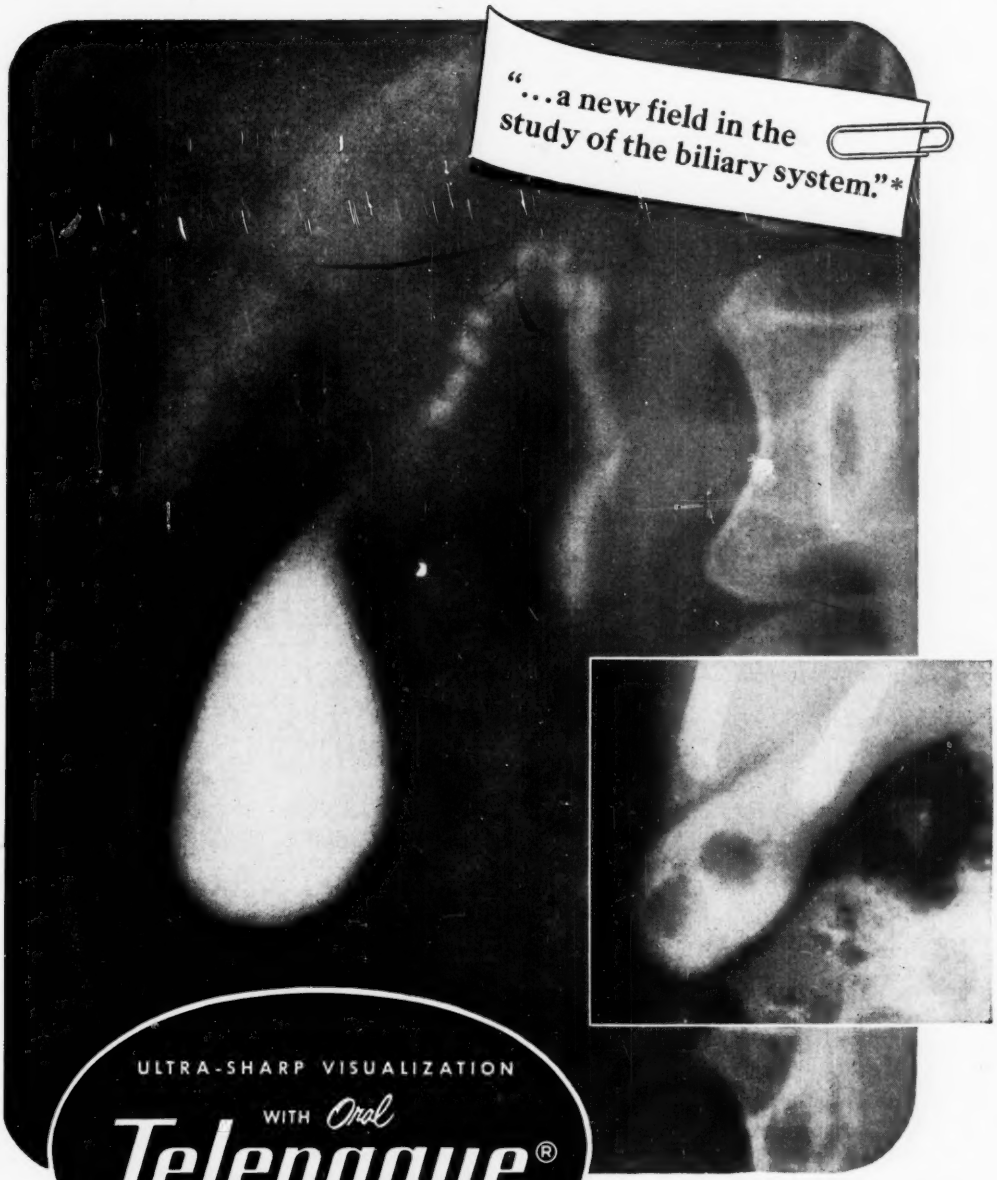

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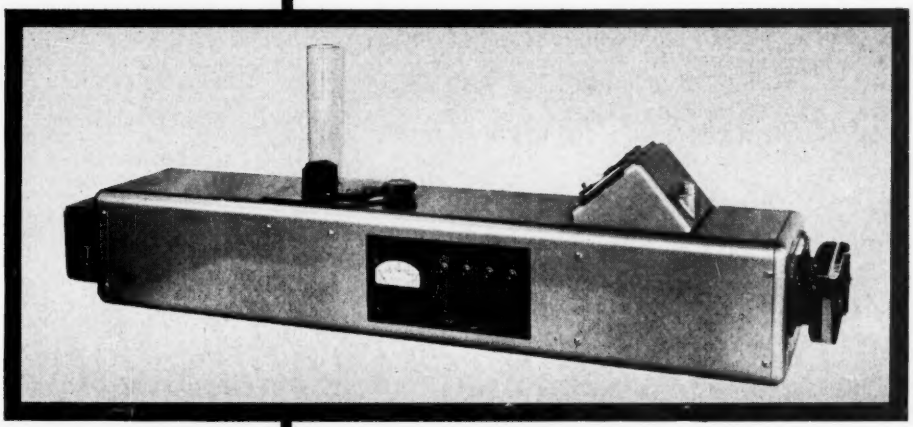

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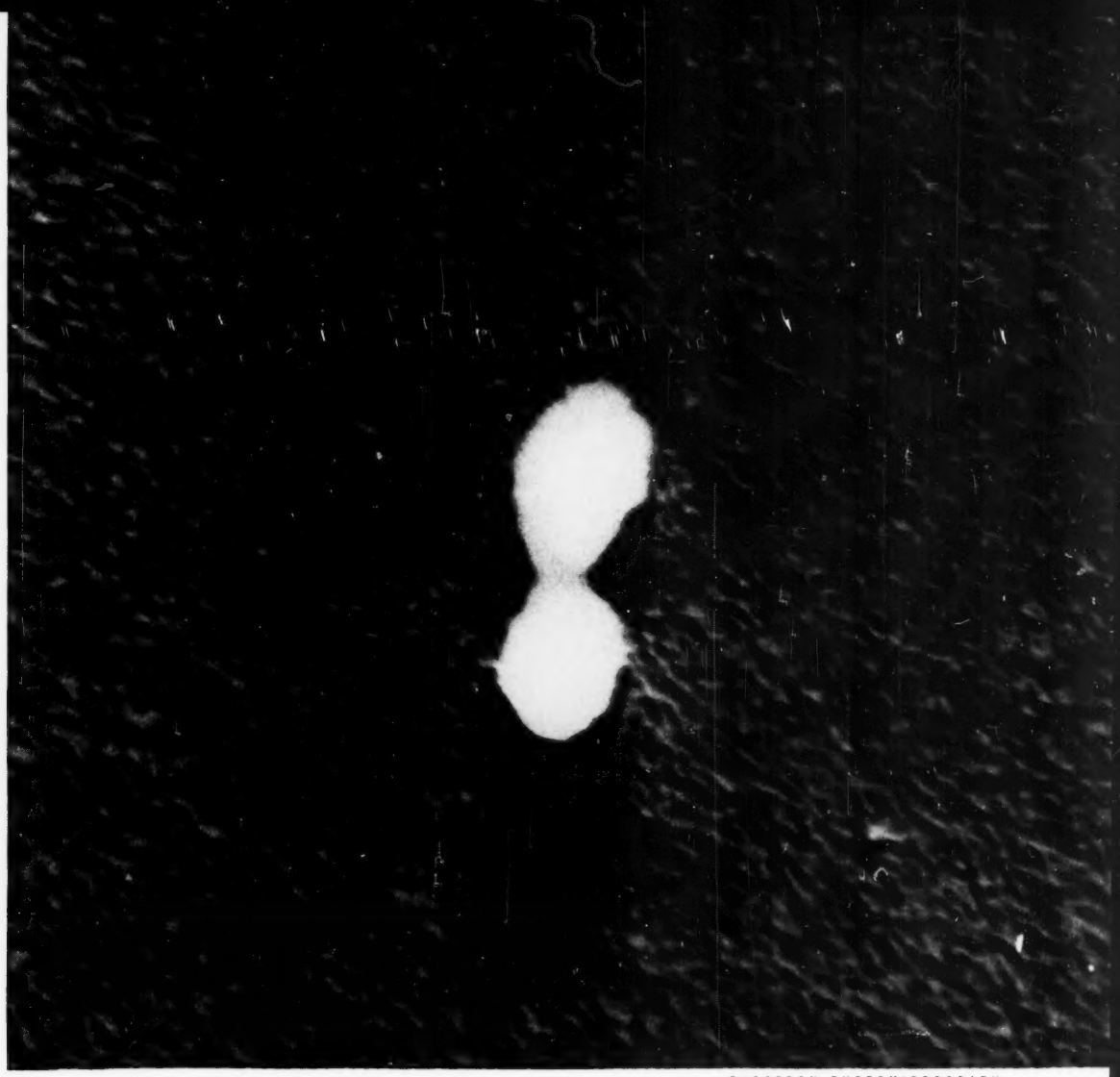

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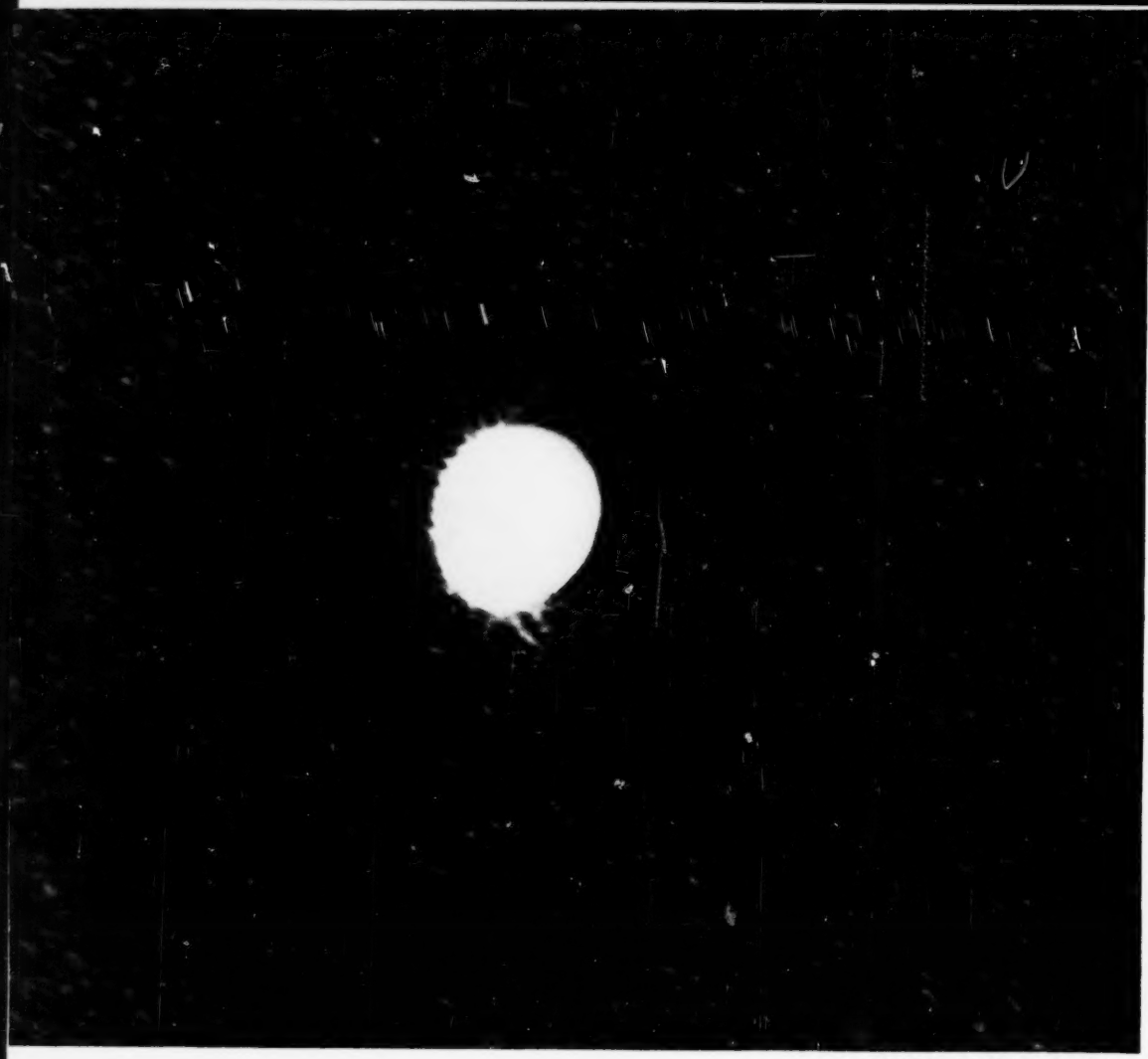

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STUDIES OF 17-HYDROXYCORTICOSTEROIDS. V. RESPONSES OF 17-HYDROXYCORTICOSTEROIDS, EOSINOPHILS, AND GLUCOSE TO ACTH AND EPINEPHRINE [1]

By ROBERT S. ELY, PATRICK F. BRAY,[2] RICHARD B. RAILE,[3] AND VINCENT C. KELLEY

(From the Lockhart Memorial Laboratory, Department of Pediatrics, University of Utah College of Medicine, Salt Lake City, Utah)

(Submitted for publication June 29, 1954; accepted July 29, 1954)

The belief has been rather widespread, because of the similarities between the responses elicited by epinephrine and ACTH, that these agents produce many of their responses by similar mechanisms. There is considerable evidence which tends to support this viewpoint: 1) Both are considered to be essential agents for adequate response of the organism to stressful situations (1–5); 2) both produce eosinopenia, lymphopenia, and depletion of adrenal ascorbic acid and cholesterol (5–10); 3) several authors have presented data indicating that epinephrine stimulation of the anterior pituitary results in release of ACTH (11–13). On the other hand, certain differences between effects of epinephrine and ACTH have been demonstrated clearly: 1) The hyperglycemic responses to these two stimulating agents are produced by different mechanisms (14, 15); 2) recent publications have suggested that this is true also of the eosinopenic response (6, 16–18); 3) the administration of epinephrine produces no increased release of 17-OH-corticosteroid hormones from the adrenal cortex such as that produced by the administration of ACTH (6, 19–21).

The apparent discrepancies among published data concerning the mechanisms of responses to these two stimulating agents have prompted further study of this problem. In the present report data are presented comparing the responses of eosinophils, blood glucose, and plasma 17-OH-corticosteroids to ACTH and to epinephrine.

MATERIALS AND METHODS

The subjects employed in this study were 40 normal children and 116 children with the following disorders: 6 with congenital adrenal hyperplasia; 25 with rheumatic disease (pre- or post-hormone therapy); 29 with mental retardation excluding mongolism and convulsive disorders; and 56 with convulsive disorders both treated and non-treated. Responses of eosinophils, glucose, and plasma 17-OH-corticosteroids to the subcutaneous injection of 0.01 mgm. epinephrine-HCl per Kg. or the intramuscular injection of 25 I.U. ACTH [4] were studied. Eosinophils were determined by the method of Randolph (22), glucose by the method of Nelson (23), and plasma 17-OH-corticosteroids by the method of Nelson and Samuels (24). Total eosinophil counts and blood glucose levels were determined at 0, 1, 2, and 4 hours. These responses are expressed as maximum per cent decrease in the eosinophil count and maximum per cent increase in glucose concentration. The 17-OH-corticosteroid values were measured at 0 and 2 hours (21).

OBSERVATIONS

Control values in the different groups

In Table I are shown the mean fasting morning values for eosinophils, glucose, and 17-OH-corticosteroids in each of the five groups of children. As shown by these data, the children with congenital adrenal hyperplasia had significantly higher eosinophil counts and lower glucose and 17-OH-corticosteroid concentrations than normal children. In patients with rheumatic disease the eosinophil counts were apparently less than normal although this difference was not significant at the 2 per cent level; their glucose and 17-OH-corticosteroid concentrations did not differ from normal. In the patients with simple mental retardation, although the mean eosinophil counts and 17-OH-corticosteroid concentrations were

[1] This work was supported in part by grants from the Life Insurance Medical Research Fund and the Helen Hay Whitney Foundation.

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[3] Present address: Department of Pediatrics, University of Minnesota, Minneapolis.

[4] ACTHAR furnished through the courtesy of the Armour Laboratories.

TABLE I

Initial fasting eosinophil counts and concentrations of glucose and 17-OH-corticosteroids in different groups of patients

Group	Eosinophils				Glucose				17-OH-corticosteroids			
	No. tests	Mean	SEM	P (*vs.* Normal)	No. tests	Mean	SEM	P (*vs.* Normal)	No. tests	Mean	SEM	P (*vs.* Normal)
Normal	56	187	±15.6	—	55	76	±2.6	—	40	12.0	±1.29	—
Congenital adrenal hyperplasia	70	265	±19.8	<.01	48	63	±2.2	<.01	18	2.7	±0.82	<.01
Rheumatic disease	59	138	±15.1	<.05 >.02	58	71	±1.9	>.05	62	14.5	±2.56	>.05
Mental retardation	29	190	±23.4	>.05	29	90	±2.2	<.01	29	8.8	±1.02	>.05
Convulsive disorder	57	188	±19.1	>.05	59	80	±1.5	>.05	52	11.1	±1.28	>.05

TABLE II

Comparison of responses of eosinophils, glucose, and 17-OH-corticosteroids to ACTH and epinephrine

Group	Eosinophils					Glucose					17-OH-corticosteroids				
	No. pts.	No. tests	Response (% *decrease*)			No. pts.	No. tests	Response (% *increase*)			No. pts.	No. tests	Response (*increase* μg. %)		
			Mean	SEM	P (*vs.* Normal)			Mean	SEM	P (*vs.* Normal)			Mean	SEM	P (*vs.* Normal)
Response to ACTH															
Normal	40	40	52	±6.6	—	40	40	12	±2.4	—	40	40	17.8	±1.76	—
Congenital adrenal hyperplasia	6	46	25	±4.5	<.01	6	32	14	±3.2	>.05	6	18	0	±0.37	<.01
Rheumatic disease	17	36	49	±7.5	>.05	15	35	14	±3.3	>.05	17	35	22.7	±4.19	>.05
Mental retardation	24	24	62	±6.3	>.05	24	24	5	±2.3	<.05 >.02	25	25	8.6	±1.63	<.01
Convulsive disorder	46	46	55	±10.9	>.05	46	46	8	±3.9	>.05	42	42	9.7	±2.80	<.02 >.01
Response to epinephrine															
Normal	20	20	54	±6.4	—	22	22	55	±6.7	—	20	20	−0.3	±1.83	—
Congenital adrenal hyperplasia	6	24	57	±4.6	>.05	6	16	86	±11.8	<.05 >.02	6	12	−0.7	±0.74	>.05
Rheumatic disease	15	26	41	±11.8	>.05	15	26	50	±7.6	>.05	15	27	0.5	±2.46	>.05
Mental retardation	5	5	64	±23.5	>.05	5	5	66	±9.8	>.05	—	—	—	—	—
Convulsive disorder	10	10	58	±10.0	>.05	10	10	53	±7.6	>.05	10	10	1.2	±1.76	>.05

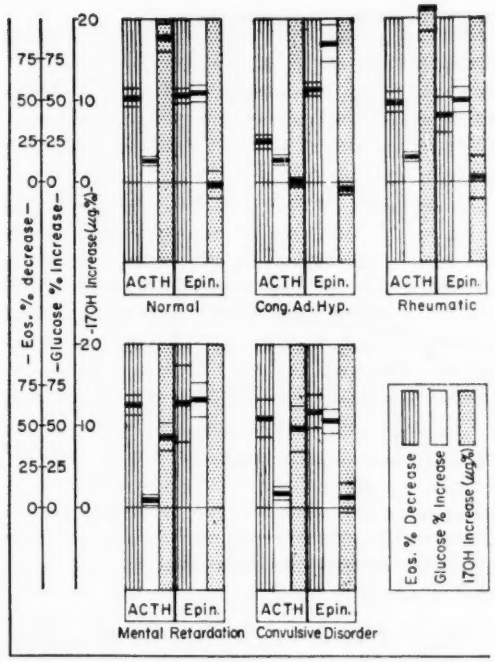

FIG. 1. MAGNITUDE OF RESPONSES IN THE DIFFERENT GROUPS

normal, the blood glucose concentrations were significantly higher than normal. The groups of patients with convulsive disorders did not differ from the normal group with respect to any of the variables studied.

Table II shows the responses of eosinophils, glucose, and 17-OH-corticosteroids to ACTH and to epinephrine in the various groups of subjects. Eosinophil responses to ACTH did not differ significantly from those of the controls in any group except that of congenital adrenal hyperplasia. In patients with this condition the eosinopenia induced by ACTH was significantly less than normal (25 per cent *vs.* 52 per cent depletion). On the other hand, the eosinopenic response to epinephrine did not differ significantly in any of the groups studied. In the congenital adrenal hyperplasia group, statistical comparison demonstrates a significant difference in the same individuals between the eosinopenic responses to ACTH and to epinephrine (ACTH: -25 ± 4.5 per cent; epinephrine: -57 ± 4.6 per cent; $p = < .01$).

The magnitude of the hyperglycemic response to a single injection of ACTH was small as compared with that to epinephrine. Among the groups studied only that with mental retardation had an hyperglycemic response to ACTH significantly less than that of the control group. In contrast, the hyperglycemic response to epinephrine was normal in this group as it was in all other groups except that with congenital adrenal hyperplasia; in the latter, this response was significantly greater than normal.

The 17-OH-corticosteroid responses to ACTH were abnormal in three of the study groups: congenital adrenal hyperplasia, mental retardation, and convulsive disorder. In the congenital adrenal hyperplasia group the initial plasma steroid levels were very low and no elevation occurred in response to ACTH. In contrast, the mental retardation and convulsive disorder groups had normal initial steroid levels but their responses to ACTH were intermediate between those of the normal and the congenital adrenal hyperplasia groups, indicating impaired but not absent adrenal cortical function. In no group was there any steroid response to epinephrine.

Figure 1 illustrates graphically the data of Table II. In the normal group eosinopenic responses to ACTH and to epinephrine were essentially the same; hyperglycemic response to epinephrine was considerably greater than that to

TABLE III

Comparison of hyperglycemic and 17-OH-corticosteroid responses to 25 I.U. ACTH vs. 100 I.U. ACTH

	Glucose (% *increase*)		17-OH-corticosteroid (μg. % *increase*)	
	ACTH		ACTH	
Pt.	25 I.U.	100 I.U.	25 I.U.	100 I.U.
T. G.	40	38	16.3	19.9
V. M.	4	9	2.4	27.6
L. W.	4	28	18.4	12.5
Z. G.	40	74	14.0	37.2
P. C.	24	18	39.2	66.1
C. B.	29	14	30.0	33.1
D. S.	37	14	37.0	66.4
T. G.	48	40	19.2	22.8
L. H.	8	11	18.4	26.7
R. S.	33	37	−11.5	27.1
C. E.	3	3	1.3	13.9
Mean	25	26	16.8	32.1
SEM	±5.1	±6.0	±4.62	±5.55
P (25 *vs.* 100 ACTH)	>0.05		<0.05 >0.02	

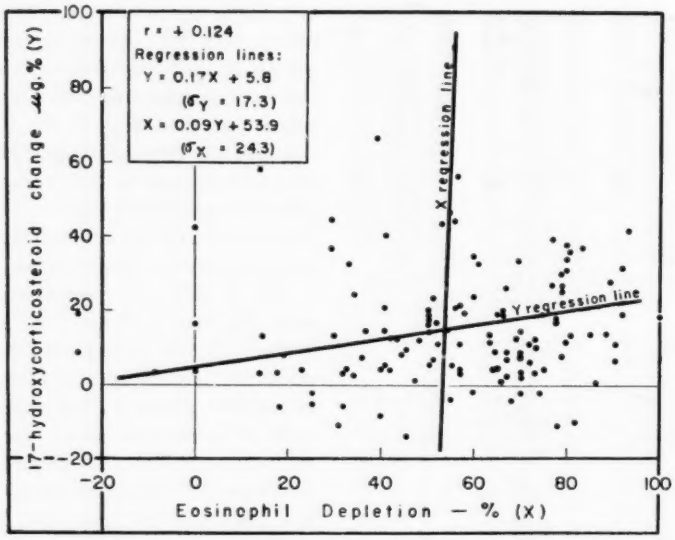

FIG. 2. COMPARISON OF EOSINOPHIL AND 17-OH-CORTICOSTEROID RESPONSES TO ACTH

ACTH; and steroid response to ACTH was of considerable magnitude whereas there was no steroid response to epinephrine. In the congenital adrenal hyperplasia group the pattern of these responses differed in that the eosinophil response to ACTH was less, the glucose response to epinephrine was greater and there was no steroid response to ACTH. Responses in the rheumatic group were comparable to those in the control group. The mental retardation group showed decreased glucose and steroid responses to ACTH in comparison with the normal group. In the convulsive disorder group the decreased steroid response to ACTH was the only response which differed from normal. Throughout all groups the glucose response to epinephrine was considerably greater than that to ACTH.

Table III compares the individual responses of glucose and of 17-OH-corticosteroids to 25 I.U. and to 100 I.U. ACTH in 11 patients. Whereas 100 I.U. ACTH produced a greater magnitude of steroid response than did 25 I.U. ACTH (32.1 *vs.* 16.8 μg. per cent) it did not produce any greater hyperglycemic response. Furthermore, there was no apparent correlation between the magnitudes of steroid and glucose responses to ACTH.

Figure 2 shows as a scattergram the comparison of simultaneous eosinopenic and steroid responses to ACTH in 128 individuals from all

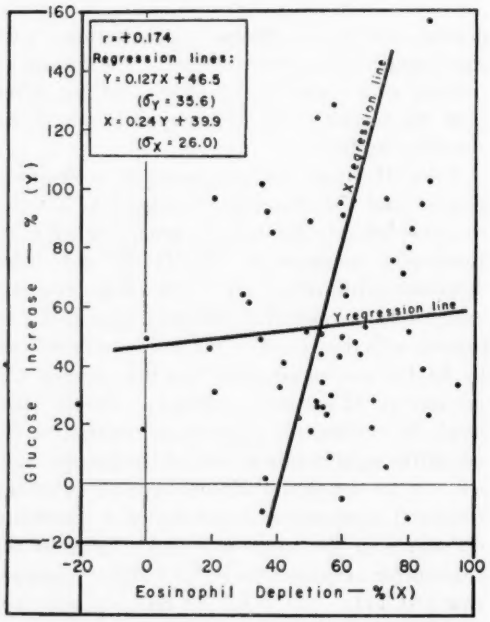

FIG. 3. COMPARISON OF EOSINOPHIL AND GLUCOSE RESPONSES TO EPINEPHRINE

groups except congenital adrenal hyperplasia. For each subject the change in steroid concentration (μg. per cent) observed two hours after the administration of ACTH was plotted against the maximum per cent decrease in eosinophil count in a four-hour period. The correlation coefficient ($\gamma = +0.124$) indicates that no significant correlation existed.

Figure 3 compares in a similar manner the simultaneous eosinopenic and hyperglycemic responses to epinephrine in 61 children. In this figure the maximum per cent decrease in eosinophil count was plotted against the maximum per cent increase in glucose concentration observed in each subject. Again, the "r" value (+ 0.174) indicates no significant correlation between these two variables.

Comparison of the ACTH-glucose response with the other responses to ACTH was not made because of the small magnitude of this response. Since no steroid response to epinephrine occurred, no comparison was made with the other responses to epinephrine.

DISCUSSION

It appears clear that the total circulating eosinophil count is influenced by adrenal cortical hormone. Eosinopenia is seen in patients with Cushing's disease (25) and in patients treated with ACTH or adrenal steroids. Eosinophilia generally exists in the adrenalectomized animal and in patients with Addison's disease (26, 27). Also, as shown here, eosinophilia of mild degree exists in children with congenital adrenal hyperplasia. These patients have a "segmental" adrenal insufficiency as shown by their inability to produce appreciable quantities of 17-OH-corticosteroids, the principal adrenal cortical hormones, even in response to large doses of ACTH.

The responses of eosinophils to epinephrine and to ACTH have been used clinically as criteria of adrenal cortical function. However, there now is considerable evidence to support the belief that the epinephrine-eosinophil response is not a valid criterion of this function (6, 7, 16, 17, 20, 28). Likewise, it has been suggested (6) that the ACTH-eosinophil response need not be mediated by an increased production of adrenal steroids. These points both are well substantiated by data presented here which show that: 1) There is no steroid response to epinephrine in any group of patients despite adequate eosinophil responses in all groups; 2) in patients with congenital adrenal hyperplasia there is some, albeit inadequate, eosinopenic response to ACTH without any concurrent steroid response; and 3) there is no significant correlation between the magnitude of eosinophil and of steroid responses to either ACTH or epinephrine. Thus, it seems that the eosinopenia produced by either epinephrine or ACTH is not related quantitatively to 17-OH-corticosteroid release by the adrenal cortex. This eosinopenia may be associated with the release of some other adrenal steroid. In addition, epinephrine induced eosinopenia may be related to some function of epinephrine other than pituitary adrenal cortical stimulation. There is evidence to suggest that it may be the result of a synergistic action between epinephrine and adrenal cortical hormones (18).

A direct relationship between the adrenal cortex and carbohydrate metabolism is known to exist. In patients with Addison's disease and in adrenalectomized dogs, episodes of hypoglycemia occur frequently. Conversely, patients with Cushing's disease often have hyperglycemia and diabetic glucose tolerance curves. Furthermore, "steroid diabetes" has been produced in animals (15) and in human subjects (29). Information such as this has led to attempts to evaluate the status of adrenal cortical function on the basis of the alterations in blood glucose concentration resulting from the administration of ACTH. Evaluation of adrenal cortical function by the hyperglycemic response to ACTH has been made: 1) In patients with hypoglycemosis (30, 31); 2) with continued ACTH administration (29, 32, 33) and; 3) in terms of the effect of ACTH on the glucose tolerance curve (29, 30). Little mention has been made of the hyperglycemic response to a single injection of ACTH. The studies reported here show that normally only a small response occurs and suggest that this test is of no practical value. The significance of the poor hyperglycemic response to ACTH seen in the group of patients with mental retardation is unknown.

It has been suggested (34) that adrenal cortical hormones participate in the alterations of

carbohydrate metabolism following the injection of epinephrine. The adequate hyperglycemic response to epinephrine without any 17-OH-corticosteroid response, observed in all the groups studied, neither supports nor refutes this concept. Even though the hyperglycemic response to epinephrine is not mediated by an increased plasma 17-OH-corticosteroid level the possibility exists that these steroids may act synergistically with the administered epinephrine in producing this hyperglycemic response in a manner similar to that postulated for the eosinopenic response to epinephrine (18). The consideration that identical synergistic mechanisms are involved has little support, since no correlation exists between the eosinopenic and hyperglycemic responses to epinephrine (Figure 3). Although the concept of an epinephrine-17-OH-corticosteroid synergism is weakened somewhat by the observations of ample hyperglycemic and eosinopenic responses to epinephrine despite very low plasma 17-OH-corticosteroid concentrations in the congenital adrenal hyperplasia group, it is not invalidated by these observations.

Responses of glucose, eosinophils and 17-OH-corticosteroids all have been used as criteria of adrenal cortical function. Since there is no significant correlation among the three responses and since the 17-OH-corticosteroid response measures adrenal cortical secretory function in terms of the major steroids produced, the latter test appears to be the one of preference. However, the clinical application of this steroid response test has certain limitations (19) and the other response tests, particularly the ACTH-eosinophil response, still have clinical values—at least as screening tests.

SUMMARY

1. Fasting levels of eosinophils, glucose and 17-OH-corticosteroids, and their responses to ACTH and to epinephrine were compared in different groups of children. Certain differences were noted among the groups studied.

2. No correlation was found between the magnitude of: 1) Eosinopenic and 17-OH-corticosteroid responses to ACTH or ; 2) eosinopenic and hyperglycemic responses to epinephrine.

3. The hyperglycemic response to ACTH was slight.

4. No 17-OH-corticosteroid response to epinephrine was found.

5. The 17-OH-corticosteroid response to ACTH is considered the most valid of the tests compared.

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REFERENCES

1. Cannon, W. B., The Wisdom of the Body. New York, W. W. Norton and Co., Inc., 1939.
2. Cannon, W. B., Newton, H. F., Bright, E. M., Menkin, V., and Moore, R. M., Some aspects of the physiology of animals surviving complete exclusion of sympathetic nerve impulses. Am. J. Physiol., 1929, **89**, 84.
3. Selye, H., The general adaptation syndrome and the diseases of adaptation. J. Clin. Endocrinol., 1946, **6**, 117.
4. Sayers, G., and Sayers, M. A., Regulation of pituitary adrenocorticotrophic activity during the response of the rat to acute stress. Endocrinology, 1947, **40**, 265.
5. Sayers, G., The adrenal cortex and homeostasis. Physiol. Rev., 1950, **30**, 241.
6. Kelley, V. C., Ely, R. S., Raile, R. B., and Bray, P. F., Comparison of eosinophil and circulating 17-hydroxycorticosteroid responses to epinephrine and ACTH. Proc. Soc. Exper. Biol. & Med., 1952, **81**, 611.
7. Speirs, R. S., Eosinopenic activity of epinephrine in adrenalectomized mice. Am. J. Physiol., 1953, **172**, 520.
8. Dowden, C. W., and Bradbury, J. T., Eosinophil response to epinephrine and corticotropin. Studies in alcoholics and nonalcoholics. J. A. M. A., 1952, **149**, 725.
9. Bergstrand, C. G., Hellström, B., and Jonsson, B., The effect of adrenocorticotropic hormone (ACTH) on the circulating eosinophils in infants. Acta paediat., 1952, **41**, 393.
10. Hills, A. G., Forsham, P. H., and Finch, C. A., Changes in circulating leukocytes induced by the administration of pituitary adrenocorticotrophic hormone (ACTH) in man. Blood, 1948, **3**, 755.
11. Harris, G. W., The hypothalamus and regulation of ACTH secretion *in* Adrenal Cortex. Transactions of the 3rd Conference 1951. E. P. Ralli, Ed., New York, Josiah Macy Jr. Foundation, 1952, pp. 54–88.
12. Hume, D. M., and Wittenstein, G. J., The relationship of the hypothalamus to pituitary-adrenocortical function. In Proc. First Clinical ACTH Conference, J. R. Mote, Ed., Phila., The Blakiston Co., 1950, pp. 134–146.

13. Farrell, G. L., and McCann, S. M., Detectable amounts of adrenocorticotrophic hormone in blood following epinephrine. Endocrinology, 1952, **50**, 274.
14. Somogyi, M., Mechanism of epinephrine—hyperglycemia. Endocrinology, 1951, **49**, 774.
15. Ingle, D. J., Production of glycosuria in the normal rat by stilbestrol and by 17-hydroxy-11-dehydrocorticosterone. Am. J. Physiol., 1941, **133**, p. 337.
16. Kark, R. M., and Muehrcke, R. C., Does adrenaline eosinopenia reflect pituitary-adrenal function in man? Lancet, 1952, **1**, 1189.
17. Best, W. R., Kark, R. M., Muehrcke, R. C., and Samter, M., Clinical value of eosinophil counts and eosinophil response tests. J. A. M. A., 1953, **151**, 702.
18. Henry, W. L., Jr., Oliner, L., and Ramey, E. R., Relationship between actions of adrenocortical steroids and adrenomedullary hormones in the production of eosinopenia. Am. J. Physiol., 1953, **174**, 455.
19. Ely, R. S., Raile, R. B., Bray, P. F., and Kelley, V. C., Studies of 17-hydroxycorticosteroids. IV. Evaluation of a standard ACTH-17-hydroxycorticosteroid response test in children. Pediatrics, 1954, **13**, 403.
20. Sandberg, A. A., Nelson, D. H., Palmer, J. G., Samuels, L. T., and Tyler, F. H., The effects of epinephrine on the metabolism of 17-hydroxycorticosteroids in the human. J. Clin. Endocrinol. & Metab., 1953, **13**, 629.
21. Raile, R. B., Ely, R. S., and Kelley, V. C., Studies of 17-hydroxycorticosteroids in children. II. Response of blood levels to the injection of certain hormones. J. Pediat., 1953, **42**, 46.
22. Randolph, T. G., Blood studies in allergy. I. The direct counting chamber determination of eosinophils in propylene glycol aqueous stains. J. Allergy, 1944, **15**, 89.
23. Nelson, N. A., Photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 1944, **153**, 375.
24. Nelson, D. H., and Samuels, L. T., A method for the determination of 17-hydroxycorticosteroids in blood; 17-hydroxycorticosterone in the peripheral circulation. J. Clin. Endocrinol. & Metab., 1952, **12**, 519.
25. Talbot, N. B., Sobel, E. H., McArthur, J. W., and Crawford, J. D., Functional Endocrinology, From Birth Through Adolescence. Cambridge, Harvard University Press, 1952, p. 243.
26. Thorn, G. W., Forsham, P. H., and Emerson, K., Jr., The Diagnosis and Treatment of Adrenal Insufficiency. 2d. ed., Springfield, Charles C Thomas, 1951, p. 54.
27. Bergenstal, D. M., and Dao, T. L. Y., Management of Addison's disease in adrenalectomized patients. Bull. New York Acad. Med., 1953, **29**, 295.
28. Speirs, R. S., and Sullivan, J., Comparison of epinephrine and ACTH action on the circulating eosinophils of mice. Am. J. Physiol., 1953, **173**, 282.
29. Conn, J. W., Louis, L. H., and Wheeler, C. E., Production of temporary diabetes mellitus in man with pituitary adrenocorticotropic hormone; relation to uric acid metabolism. J. Lab. & Clin. Med., 1948, **33**, 651.
30. McQuarrie, I., Bauer, E. G., Ziegler, M. R., and Wright, W. S., The metabolic and clinical effects of pituitary adrenocorticotrophic hormone in spontaneous hypoglycemosis. Proc. First Clin. ACTH Conference, J. R. Mote, Ed., Philadelphia, The Blakiston Co., 1950, p. 225.
31. McQuarrie, I., Spontaneous hypoglycemia: Clinical and metabolic studies *in* Carbohydrate Metabolism, a symposium on the clinical and biochemical aspects of carbohydrate utilization in health and disease. V. A. Najjar, Ed., Baltimore, The Johns Hopkins Press, 1952, p. 76–91.
32. Kinsell, L. W., Margen, S., Michaels, G. D., Reiss, R., Frantz, R., and Carbone, J., Studies in fat metabolism. III. The effect of ACTH, of cortisone, and of other steroid compounds upon fasting-induced hyperketonemia and ketonuria. J. Clin. Invest., 1951, **30**, 1491.
33. Ingle, D. J., Winter, H. A., Li, C. H., and Evans, H. M., Production of glycosuria in normal rats by means of adrenocorticotrophic hormone. Science, 1945, **101**, 671.
34. Winternitz, W. W., and Long, C. N. H., Participation of adrenal cortex in alterations in carbohydrate metabolism produced by epinephrine. Proc. Soc. Exper. Biol. & Med., 1952, **81**, 683.

THE DISAPPEARANCE RATE OF COLLOIDAL RADIOGOLD FROM THE CIRCULATION AND ITS APPLICATION TO THE ESTIMATION OF LIVER BLOOD FLOW IN NORMAL AND CIRRHOTIC SUBJECTS

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The bromsulphalein method of estimating liver blood flow in man first introduced by Bradley, Ingelfinger, Bradley, and Curry (1) has proved to be a very useful tool in the hands of various workers. Though there is not complete agreement as to the question of extrahepatic dye loss and the problem of a single hepatic vein sample not being entirely representative of all the blood leaving the liver, a considerable amount of information has been gathered by studying the state of liver circulation in normal and diseased subjects and its alterations produced by exercise, posture, anesthesia, and various drugs. However, the technic employed is by no means a simple one and requires the team work of several highly trained people.

Very recently, an entirely different method has been applied to the study of hepatic circulation. It is based on the assumption that the phagocytes of liver and spleen are highly efficient in removing colloid particulate matter from the blood stream in a single passage. If this assumption holds true, then the disappearance rate of this colloid from the circulation would essentially be a parameter of liver blood flow. Since the liver and spleen are in series with each other and since the bone marrow takes up only minimal amounts of this colloid, it was felt by Sheppard, Jordan, and Hahn (2) that it should be possible to calculate liver blood flow from the disappearance rate constant as the fraction of blood volume perfusing the liver per unit time. This idea has been put into practice by Dobson, Warner, Finney, and Johnston (3) who used chromic phosphate labeled with radiophosphorus as the particulate matter. However, this method again appears not to be as simple as one would want it to be for a routine diagnostic procedure. The preparation of a chromic phosphate colloid of suitable particle size is quite a laborious procedure, and the determination of the disappearance rate itself involves the withdrawal of serial blood samples which, to the patient, results in the loss of a considerable amount of blood.

A further simplification of the estimation of liver blood flow in man would, therefore, be afforded by using a colloid readily at hand, and by labeling it with a γ-emitting isotope which would permit external determination of the disappearance rate without the need for withdrawing blood samples. The present paper reports some results obtained with colloidal radioactive gold (Au^{198}) used as the particulate matter, the measurements being carried out by means of a counter placed between the calves of the subject. Various factors which might influence the reliability and accuracy of this method as well as some of the questions which have to be answered before the transformation of colloid disappearance rates into terms of liver blood flow could be considered to be justified, will be discussed. Some preliminary results have been published elsewhere (4).

METHODS

Colloidal radioactive gold. Colloidal Au^{198} was obtained from A.E.R.E., Harwell, England, as a 0.5 per cent (W/V) solution with a specific activity of between 10 and 20 mc. per ml. The average particle size of this preparation, estimated by electron microscopic observation, is about 250 Å and the scatter around the mean is very small.[1] After suitable dilution with sterile pyrogen-free isotonic saline, between 25 and 250 μgm. of colloidal gold containing between 50 and 100 μc. Au^{198} were injected intravenously. Care was taken to avoid any effect of dilution on colloid stability.

Blood samples. In the early series of experiments which were done in order to obtain some information on the reliability of external measurements and on the influence of mixing of the colloid particles within the cir-

[1] We wish to thank Mr. F. Hudswell, Chemist in Charge of Chemical Production Group, Isotope Division, A.E.R.E., Harwell, England, for kindly providing this information.

culating blood on the disappearance rate, it was necessary to withdraw serial blood samples for determination of radioactivity and T-1824 concentration. An indwelling needle was placed into the brachial artery of the other arm and was left there throughout the experiment. By removing the stylet, 6.0 ml. samples were taken into heparinized tubes at the following times after injection: 1, 2, 3, 4, 5, 7, 9, 12, 15, 20, 25, 35, 45, and 60 minutes.

Determination of radioactivity. The radioactivity of the blood samples was determined in the liquid counter designed by Veall (5), 1.0 ml. of blood having been hemolyzed and diluted with 10 ml. of tap water. External measurements were done using an unshielded lead cathode γ-counter (20th Century G.10.Pb) placed between the calves of the patient. In preliminary experiments the increase in the background count due to the accumulation of the radiogold in the liver and spleen was found to be negligible. With both methods, the absolute counting rate was always higher than twice the background even at the end of one hour following injection.

Plasma volume. Between 10 and 15 mgm. of purified T-1824 (I.C.I.) as a 0.075 per cent (W/V) solution were injected simultaneously with the radiogold injection into another vein of the same arm. Dye concentrations were determined with a Zeiss photoelectric spectrophotometer at a wave length of 6200 Å. In the early series of patients where serial blood samples were taken, the values obtained were plotted on semilogarithmic paper and extrapolated back to time zero. In the later series where the colloid disappearance rate was determined by external counting, only a ten-minute sample was withdrawn and a 1.5 per cent loss of dye was assumed to have occurred during this period. The standard amount of dye was delivered to a blank sample by a carefully calibrated 50 μl. pipet.

Hematocrit. Two 1 ml. samples of heparinized blood taken simultaneously with the blank samples, were pipeted into Wintrobe tubes with a 2 mm. bore. They were spun for 30 min. at 3000 rpm. in an ordinary centrifuge applying a force of 1500 × gravity. A correction for the amount of plasma trapped between the red cells was applied to the mean of both readings according to the graph published by Chaplin and Mollison (6).

Blood volume. Total blood volume was calculated from the plasma volume and the corrected venous hematocrit after the latter had been further corrected for the uneven distribution of red cells and plasma within the circulation by applying a correction factor of 0.91. This factor was shown by Chaplin, Mollison, and Vetter (7) to be valid over a wide range of hematocrits.

RESULTS

Calculation of the disappearance rate constant

When plotted on semilogarithmic paper the actual disappearance curve of the colloidal gold appears to be the composite of a series of simple exponential curves. The first, *i.e.*, fastest, component of this curve is obtained by the usual graphical separation of the single components, and only this component is taken for calculation of the disappearance rate constant k. A thorough description of this procedure has been given by Dobson and Jones (8) as well as in a previous paper by the present authors (4).

Theoretically, mixing of the injected particulate matter within the circulating blood should be essentially completed before the values obtained are used for the determination of the half time of disappearance. It appears from the T-1824 curves that this is not always the case. Dobson, Warner, Finney, and Johnston (3) have tried to overcome this difficulty by expressing the sample data as a ratio of colloid concentration over dye concentration, thus accounting for mixing influences which may occur during the first few minutes following injection.

The value of k obtained by using this ratio may sometimes appreciably differ in either direction from the rate constant calculated from the colloid disappearance curve alone. However, on the average this difference is almost negligible, and this has been shown to be true even in cases with impaired liver function (4). Therefore, it was felt that the serial determination of T-1824 concentrations is not essential for the accuracy of this method and accordingly, all values of the rate constant k in the accompanying tables should be understood as simple colloid disappearance rate constants.

The validity of external measurements

In five subjects the disappearance rate constants obtained from blood samples and by ex-

TABLE I

Comparison of disappearance rates obtained from blood samples and from the calves

Case	Name	Diagnosis	k_{blood} ($min.^{-1}$)	k_{calves} ($min.^{-1}$)	k_{ca}/k_{bl}
1	SCHI	Cirrhosis	.066	.065	.985
2	LAM	Hepatitis	.141	.141	1.000
3	KOR	Cardiac failure	.151	.153	1.013
4	ALT	Hypertension	.190	.201	1.053
5	ALB	Normal	.248	.267	1.079
Mean					1.027

ternal measurement have been compared (Table I). On the average, the constants determined between the calves were slightly higher than those obtained from blood samples. However, this difference was only 2.7 per cent and cannot be considered to be significant. Thus, external measurement is regarded to be a satisfactory method of determining the removal rate of the colloidal gold from the blood stream. Moreover, the values obtained are representative for both arterial and venous blood concentrations and, therefore, the differences which have been shown by Dobson, Warner, Finney, and Johnston (3) to exist between both concentrations due to mixing influences, are eliminated.

Reproducibility of results

In another group of five subjects a second estimation of the disappearance rate constant was done one hour after the first, with a dose of colloidal gold not exceeding 100 μgm. At this time, the background count between the calves due to the radioactive material still circulating in the blood and presumably consisting of very small particles, was considered to be fairly stable. It had been found in preliminary experiments that during the second hour following injection the disappearance rate of these particles has never been higher than a few per cent per hour. There was, on the average, no difference between both series (Table II). The differences in individual cases have been very small. It is, therefore, concluded that the reproducibility of the results obtained with this method is satisfactory.

Effects of quantity of particulate material injected

In a further group of five subjects the disappearance rate constant was determined by injecting 250 μgm. of colloidal radiogold. In three of these, the experiment was repeated after one hour with six times this amount of material. In two cases, twice the amount was given. Removal of Au^{198} on second injection was definitely lowered in the first three cases, whereas in the other two cases no significant difference was observed (Table III). This "overloading" effect was not to be expected since Dobson and Jones in their experiments on mice (8) had used comparatively much larger quantities of chromic phosphate.

It is, however, conceivable that the magnitude of this "overloading" effect does not depend as much on the weight of the particulate material injected as on the number of particles. Since the preparation used in the present experiments contained, presumably, at least ten times as many particles per unit weight as the chromic phosphate preparation used by Dobson and co-workers, apparently the phagocytes of the liver are unable to handle all the particles passing through the liver capillaries in a given time.

It may, therefore, be advisable not to increase the quantity of colloidal gold over 250 μgm. This has not been done in the studies reported below. In fact, most of the subjects studied were given quantities below 100 μgm.

TABLE II

Results of repeated measurements between the calves

Case	Name	Diagnosis	k_1 ($min.^{-1}$)	k_2 ($min.^{-1}$)	k_2/k_1
1	BUR	Posthepatitis	.178	.182	1.022
2	FRA	Posthepatitis	.216	.210	.972
3	NOT	Normal	.224	.224	1.000
4	WUD	Mitral stenosis	.235	.243	1.034
5	WAW	Normal	.292	.286	.979
Mean					1.001

TABLE III

Effects of increasing the quantity of stable colloidal gold on the disappearance rate

Case	Name	Diagnosis	k_1 ($min.^{-1}$)	k_2 ($min.^{-1}$)	k_2/k_1
1	KOR	Cardiac failure	.152*	.132†	.868
2	ALT	Hypertension	.195*	.139†	.713
3	SIM	Normal	.276*	.243†	.881
4	BER	Normal	.252*	.256‡	1.016
5	TOR	Normal	.314*	.309‡	.984

* Amount injected: 0.25 mgm.
† Amount injected: 1.50 mgm.
‡ Amount injected: 0.50 mgm.

Efficiency of removal

It is clearly essential to determine to what extent the liver phagocytes are capable of clearing the blood passing through the capillaries completely of all the particles within a single passage. If this is the case the disappearance rate constant should then represent the fraction of blood volume perfusing the liver per unit time and, if the blood

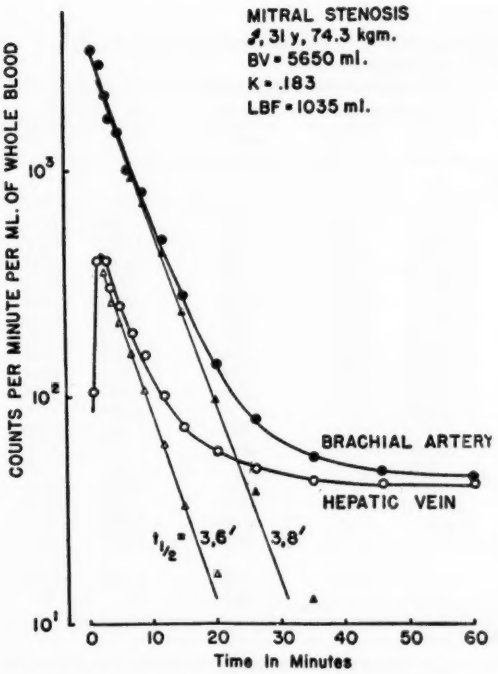

FIG. 1. RADIOGOLD CONCENTRATIONS IN HEPATIC VEIN AND BRACHIAL ARTERY BLOOD FOLLOWING INTRAVENOUS INJECTION OF COLLOIDAL RADIOGOLD IN A CASE OF MITRAL STENOSIS WITH SLIGHTLY ELEVATED PULMONARY ARTERY PRESSURES BUT WITHOUT EVIDENCE OF CARDIAC FAILURE

The fastest component of both curves have been separated graphically by successive subtraction of the slower components from the actual values. The decrease in radioactivity is due to the removal of the large and medium-sized particles by the liver phagocytes. In this case, about 13 per cent of the particles passed the liver without having been picked up during the first passage. As the fraction of very small particles increases with time, liver vein and peripheral activity equal each other.

volume is known, the liver blood flow per minute can be calculated.

To investigate the efficiency of removal of the particles a Cournand catheter was placed into a right liver vein by the usual technic in three patients (one with and two without impaired liver cell function), and serial blood samples were taken from there as well as from the brachial artery. Allowance was made for the delay in appearance of the hepatic vein blood at the other end of the catheter. During the first five minutes following the injection the ratio of the radiogold concentrations of the hepatic vein to the brachial artery blood at the same time was found to be constant with values of 13, 22, and 19 per cent, respectively. The average value of about 18 per cent is in striking agreement with the results of animal experiments done with chromic phosphate (8) and with those of liver perfusion experiments with radiogold performed by Little and Kelly (9).

This ratio increases with time due to the relative increase of very small particles still present in the circulation and presumably never to be picked up by the reticulo-endothelial cells of the liver. At the end of one hour following injection the radioactivity in the hepatic vein samples almost equals that in peripheral blood. If the usual corrections are applied to both disappearance curves, then the first and fastest components of both curves run parallel to each other (Figure 1).

These results would indicate that the colloid disappearance methods regardless of the type of particulate matter used, underestimate the liver blood flow by almost 20 per cent, and that they measure a minimal rather than the true liver blood flow.

The disappearance rate constant in normal subjects

Eighteen male and seven female patients without any apparent liver or circulatory disorders were selected for determinations of the colloidal radiogold disappearance rate and calculations of minimal liver blood flow, the results of which are presented in Table IV. Hematocrits, plasma volumes (Mean: 43.5 ml. per Kg.), and blood volumes could be considered to be normal. It should be noted that there was a wide distribution of body weights.

The disappearance rate constant in liver cirrhosis

Colloid disappearance rates were determined and minimal liver blood flows were calculated in 12 patients—10 males and 2 females— and the results are given in Table V. According to their laboratory findings (galactose excretion, thymol turbidity, zinc sulfate turbidity, alkaline phosphatase, cephalin flocculation, prothrombin time, bromsulphalein-clearance and -retention, serum protein electrophoresis) and to the clinical picture, all patients were suffering from marked

TABLE IV

Data on blood volume, disappearance rate constant, and minimal liver blood flow from 18 male and 7 female patients without apparent liver or circulatory disorders

Case	Name	Weight (*Kg.*)	Corr. ven. hematocr. (%)	Plasma volume (*ml.*)	Blood volume (*ml.*)	Blood volume (*ml./Kg.*)	Disappear. rate const. ($min.^{-1}$)	Liver blood flow (*ml.*)	Liver blood flow (*ml./Kg.*)
				Males					
1	SCHW	95.7	44.1	4,580	7,650	79.9	.178	1,360	14.2
2	BAR	69.5	46.7	3,070	5,350	77.0	.210	1,120	16.1
3	KAR	73.6	43.0	3,360	5,520	75.0	.227	1,255	17.0
4	LAC	70.5	46.9	3,540	6,180	82.6	.227	1,405	19.9
5	LEI	73.5	50.0	2,970	5,450	74.1	.243	1,320	15.6
6	WEN	70.9	39.7	3,070	4,810	68.0	.248	1,195	16.8
7	KRA	84.5	44.0	3,190	5,310	63.0	.248	1,320	15.6
8	BER	96.2	41.0	4,750	7,590	78.8	.252	1,910	19.9
9	NIK	90.2	46.8	3,910	6,800	75.4	.252	1,710	19.0
10	LUK	87.2	45.3	3,340	5,860	67.4	.256	1,500	17.2
11	MIT	73.3	40.6	3,830	6,080	83.0	.256	1,560	21.3
12	WAW	64.9	40.4	2,860	4,520	69.8	.289	1,305	20.2
13	MOD	59.5	40.5	2,920	4,620	77.7	.289	1,380	23.2
14	ASB	60.5	44.9	2,260	3,820	63.1	.301	1,150	19.0
15	MUL	52.3	38.2	2,680	4,100	78.5	.308	1,260	24.1
16	STO	87.9	39.4	3,550	5,530	62.9	.322	1,780	20.2
17	SCHU	51.0	41.6	2,790	4,490	78.8	.356	1,600	28.1
18	MAD	77.1	42.0	3,220	5,210	67.8	.385	2,010	26.1
Mean		74.7				73.5	.269	1,425	19.5
±(M)							.012		0.9
				Females					
1	PRO	42.6	36.1	2,170	3,230	75.8	.193	625	14.7
2	SIC	66.5	42.5	2,940	4,800	72.1	.213	1,020	15.4
3	GAM	70.4	38.2	2,980	4,570	64.9	.213	975	13.9
4	NOT	46.5	39.9	2,080	3,270	70.3	.224	735	15.8
5	ALB	55.2	39.6	2,480	3,880	70.3	.267	1,035	18.7
6	ENE	54.6	40.1	2,810	4,420	80.1	.283	1,250	22.9
7	TSC	67.3	41.1	2,960	4,720	70.2	.301	1,420	21.1
Mean		57.6				71.9	.242	1,010	17.5
±(M)							.059		4.1
				Males and Females					
Mean		69.9				73.1	.262	1,310	19.0
±(M)							.010		0.8

cirrhosis of the liver, most of them presenting an outspoken history of chronic alcoholism. However, in none of the patients could appreciable amounts of free abdominal fluid be demonstrated.

DISCUSSION

An evaluation of the usefulness of the colloid disappearance rate as a tool in the diagnosis of liver and circulatory disorders would have to concentrate on the following questions:

1. Does the disappearance rate reflect the state of liver circulation or is it affected by factors primarily unrelated to liver blood flow, *e.g.*, the function of the liver cell?

2. If there are such factors, is the degree of their influence on the disappearance rate dependent on the type or intensity of the liver or circulatory disorder present?

3. If it should appear to be justified to take the disappearance rate as a parameter of the state of liver circulation, is the present technic reliable and simple enough in order that it could be introduced as a routine clinical procedure?

1. If this method should permit an estimation of liver blood flow then the values obtained in

TABLE V

Data on blood volume, disappearance rate constant, and minimal liver blood flow from 10 male and 2 female patients with liver cirrhosis

Case	Name	Weight (Kg.)	Corr. ven. hematocr. (%)	Plasma volume (ml.)	Blood volume (ml.)	Blood volume (ml./Kg.)	Disappear. rate const. ($min.^{-1}$)	Liver blood flow (ml.)	Liver blood flow (ml./Kg.)
				Males					
1	EIC	67.5	30.2	4,450	6,140	91.0	.051	315	4.65
2	SCHI	67.1	42.5	3,220	5,250	78.0	.066	345	5.15
3	MAL	59.5	29.6	3,440	4,710	79.3	.074	350	5.9
4	KUM	65.5	40.0	3,620	5,690	84.6	.081	460	6.85
5	SEJ	64.9	38.3	4,280	6,560	101.1	.084	550	8.45
6	STE	60.5	43.0	3,610	5,940	98.1	.086	510	8.45
7	SIE	71.4	43.6	3,420	5,670	79.8	.107	605	8.5
8	POS	84.1	38.2	4,330	6,640	77.8	.117	775	9.25
9	WAY	63.0	35.6	3,540	5,240	83.1	.131	685	10.9
10	ZIE	55.0	35.0	2,925	4,290	78.0	.143	610	11.1
Mean		65.9				85.1	.094	520	7.92
				Females					
1	PRA	95.4	43.0	3,920	6,450	67.6	.076	490	5.15
2	HOF	49.6	35.2	3,020	4,450	89.6	.129	575	11.6
Mean		72.5				78.6	.103	535	8.38
				Males and Females					
Mean		66.9				84.0	.095	525	8.0

normals should, at least to a certain extent, agree with values already obtained by other methods whose accuracy has been reasonably established. That this is the case is shown in Table VI where several data are listed for convenience of comparison. It may be calculated that the average of all data obtained with the bromsulphalein method in normal men and women is about 1410 ml. per min., which is only about 100 ml. per min. higher than the average of the radiogold results. Furthermore, the average value of 19.0 ml. per min. per Kg. body weight is certainly within the range of the data reported by other groups. This despite the fact that we are bound to assume that the colloid methods underestimate the liver blood flow to an appreciable extent.

The radiogold results in cirrhotic patients are in agreement with the data previously given by

TABLE VI

Average normal liver blood flow values given by various authors

Authors	Ref.	Sex	Number of cases	LBF (ml./min.)	Method used
Bradley, Ingelfinger, Bradley, and Curry	(1)	M, F	23	1,510	BSP
Bradley	(10)	M	50	1,490	BSP
Bradley, Ingelfinger, and Bradley	(11)	M	73	1,580	BSP
Sherlock, Bearn, Billing, and Paterson	(12)	M, F	32	1,400	BSP
Wilkins, Culbertson, and Rymut	(13)	M, F	21	1,380	BSP
Shackman, Graber, and Melrose	(14)	M, F	18	1,260	BSP
Myers	(15)	M, F	9	1,390	BSP
		M, F	10	1,780	Urea
Bondy, James, and Farrar	(16)	M, F	7	1,470	BSP and urea
Dobson, Warner, Finney, and Johnston	(3)	M	29	1,790	Chromic phosphate
Present authors		M	18	1,425	Colloidal gold
		F	7	1,010	Colloidal gold

Bradley, Ingelfinger, and Bradley (11) in men and by Bollman, Khattab, Thors, and Grindlay (17) in dogs. In some instances they are even considerably lower.

There remains the possibility that not only the function of the liver cells but also the removing ability of the phagocytes is impaired in liver disease; however, we are not aware of any conclusive evidence in the literature which would prove this point. In separate experiments the results of which have been published elsewhere (18) we have carefully compared the blood clearance rates of colloidal radiogold and of bromsulphalein in patients with various degrees of liver cirrhosis. Though the clearance of bromsulphalein is, of course, strongly dependent on liver blood flow it also depends on liver cell function. If the colloid clearance depended on liver cell function some correlation between the two clearance rates might have been expected. However, no correlation at all was found. It has been even possible to define an "extraction factor" of bromsulphalein by calculating the ratio of the amounts of blood which are cleared by both substances per minute, in order to eliminate the influence of liver blood flow on the bromsulphalein clearance and thereby to obtain a clearer indication of the function of the liver cells. This factor showed large variations with varying degrees of cirrhosis and it is, therefore, highly probable that phagocyte function and liver cell function are independent of each other. Moreover, the inability to handle large amounts of particulate material was equally shared by normal subjects and cases with impaired liver cell function (Table III), and in the group of patients who were subjected to catheterization studies the case who showed the highest efficiency of removal was that whose liver function was definitely impaired.

2. Apart from the factor of removal efficiency of the phagocytes there is, however, another factor whose significance is rather difficult to assess. An underestimation of liver blood flow might also result from the presence of arterio-venous or veno-venous anastomoses through which a certain fraction of the blood entering the hepatic circulation might by-pass the liver capillaries. This factor might be quite dependent on the intensity of the liver disorder. It is, for instance, fairly conceivable that the magnitude of these shunts is considerably increased in the presence of liver cirrhosis (19) and that, therefore, liver blood flow in such cases is grossly underestimated by the colloid methods. It should be noted, however, that these objections are also valid with methods like the bromsulphalein technic which are based on the Fick principle.

3. Assuming that the colloid disappearance rate reflects the state of liver circulation this

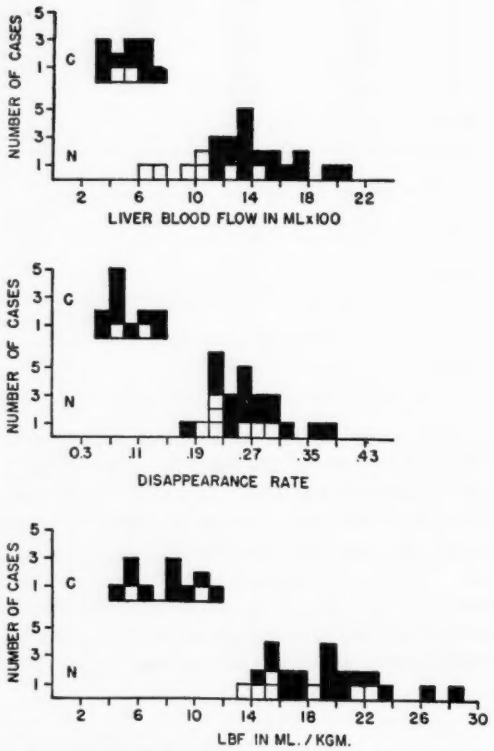

FIG. 2. COMPARISON OF THE RESULTS OBTAINED IN NORMAL (N) AND CIRRHOTIC (C) PATIENTS EXPRESSED IN THREE WAYS

There is a considerable overlap between both groups when the minimal liver blood flows are expressed in terms of absolute flows. The average minimal liver blood flow in female subjects (open squares) is smaller than in males (full squares) because of their smaller blood volume. A satisfactory separation between normal and cirrhotic values can be achieved by either relating the absolute amounts of flow to body weight or by simply using the disappearance rate constants. Large individual variations are observed but there is now no significant difference between normal male and female subjects.

method should be able to provide clinically useful results. Dobson and coworkers have pointed out that the disappearance rate constant is a more convenient term for describing the state of hepatic circulation than the quantitative flow expressed in liter per minute. This view is confirmed by the present observations. In normal subjects with small blood volumes very often liver blood flows below 1000 ml. per min. may be encountered, and this tends to obscure the significance of low values found in cases with liver disease. Relating the liver blood flow to either body weight or body surface area leads to a better separation between values obtained in normal and cirrhotic patients; however, the separation between the disappearance rate constants is equally satisfactory (Figure 2), and the scatter in normal subjects is about the same (Table IV). It is, therefore, felt that the method of estimating liver blood flow and then expressing it in terms per Kg. body weight or m^2. surface area does not constitute any considerable advantage over the use of the disappearance rate constant alone. On the contrary, the omission of blood volume determination would further simplify the procedure to a considerable extent.

It appears from the rough agreement between the averages of disappearance rate constants obtained with chromic phosphate and with colloidal gold that the factor of particle size does not play that important role which was ascribed to it by Dobson and Jones (8). This was to be expected from the data published by Zilversmit, Boyd, and Brucer (20). It was shown by this group that very small particles are, indeed, very slowly removed from the circulation but that the decrease in clearance times is very small when particles larger than 270 Å in diameter are used. Moreover, the effect of the small fraction of minute particles in the preparation on the disappearance curve is, in any case, corrected for by the graphical separation of the fastest component. Therefore, the use of a preparation like the chromic phosphate colloid which is so difficult and time-consuming to produce, is most probably not essential for the accuracy of this method. The small difference in the averages of both colloid disappearance rates might well be due to the fact that we were unable to assemble such a satisfactory group of young men between 21 and 26 years of age in an apparently enviable state of health.

With the present amount of radiogold injected (50 to 100 μc.) not more than 5 rep. are delivered to the liver or spleen. In fact, several hundred times this dose of Au^{198} had been administered in this laboratory to patients with chronic myelocytic leukemia without any subsequent impairment of liver function (21). The radiation dose could be even further reduced by using a more sensitive counting equipment, such as encircling one of the patient's legs with a multiple counter arrangement of the type which has been developed by Veall, Vetter, and Baptista (22, 23).

Colloidal gold with a suitable particle size and a high specific activity is available in large quantities which in most hospitals where radioisotopes are used, are always more or less on stock for therapeutic purposes. Being a γ-emitter, it permits convenient determination of its removal rate from outside the body. The procedure is fairly simple and could even be more simplified by the use of a logarithmic counting rate meter in combination with an automatic recording instrument.

In conclusion, it appears in the light of present knowledge that the colloid disappearance rate is, to a large extent, related to the state of liver circulation. The actual value of liver blood flow calculated from the disappearance rate constant and the blood volume must be treated with reserve, in view of the fact that the liver does not clear the blood completely in a single passage. However, its inaccuracy is probably not greater than that of other methods currently in use for the estimation of various hemodynamic data, such as cardiac output or renal blood flow. The procedure of determining the colloid disappearance rate is simple, reliable, and harmless to the patient.

SUMMARY

1. The disappearance rate of small amounts of colloidal radioactive gold has been used to calculate the minimal fraction of the blood volume perfusing the liver per minute.

2. The average value for the disappearance rate constant obtained in 18 normal men was 0.269 and in 7 normal women 0.242 $min.^{-1}$, correspond-

ing to an average minimal liver blood flow of 1425 and 1010 ml. per min., respectively.

3. In 12 cirrhotic patients a grossly diminished disappearance rate was observed.

4. The results of several experiments carried out in order to determine the reliability and accuracy of this method have been discussed.

REFERENCES

1. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J., The estimation of hepatic blood flow in man. J. Clin. Invest., 1945, **24**, 890.
2. Sheppard, C. W., Jordan, G., and Hahn, P. F., Disappearance of isotopically labeled gold colloids from the circulation of the dog. Am. J. Physiol., 1951, **164**, 345.
3. Dobson, E. L., Warner, G. F., Finney, C. R., and Johnston, M. E., The measurement of liver circulation by means of the colloid disappearance rate. I. Liver blood flow in normal young men. Circulation, 1953, **7**, 690.
4. Falkner, R., Neumayr, A., and Vetter, H., Die Bestimmung des Blutflusses durch die Leber mit kolloidalem Radiogold. Symposium on clinical applications of radioactive isotopes, Bad Gastein, January 1954, Strahlenther., in press.
5. Veall, N., A Geiger-Muller counter for measuring the beta-ray activity of liquids, and its application to medical tracer experiments. Brit. J. Radiol., 1948, **21**, 347.
6. Chaplin, H., Jr., and Mollison, P. L., Correction for plasma trapped in the red cell column of the hematocrit. Blood, 1952, **7**, 1227.
7. Chaplin, H., Jr., Mollison, P. L., and Vetter, H., The body/venous hematocrit ratio: its constancy over a wide hematocrit range. J. Clin. Invest., 1953, **32**, 1309.
8. Dobson, E. L., and Jones, H. B., The behavior of intravenously injected particulate material: its rate of disappearance from the blood stream as a measure of liver blood flow. Acta med. Scandinav., 1952, **144**, suppl. 273.
9. Little, R. C., and Kelly, H. B., Removal of radioactive gold colloid by the perfused mammalian liver. Am. J. Physiol., 1953, **173**, 265.
10. Bradley, S. E., Variations in hepatic blood flow in man during health and disease. New England J. Med., 1949, **240**, 456.
11. Bradley, S. E., Ingelfinger, F. J., and Bradley, G. P., Hepatic circulation in cirrhosis of the liver. Circulation, 1952, **5**, 419.
12. Sherlock, S., Bearn, A. G., Billing, B. H., and Paterson, J. C. S., Splanchnic blood flow in man by the bromsulfalein method: the relation of peripheral plasma bromsulfalein level to the calculated flow. J. Lab. & Clin. Med., 1950, **35**, 923.
13. Wilkins, R. W., Culbertson, J. W., and Rymut, A. A., The hepatic blood flow in resting hypertensive patients before and after splanchnicectomy. J. Clin. Invest., 1952, **31**, 529.
14. Shackman, R., Graber, I. G., and Melrose, D. G., Liver blood flow and general anaesthesia. Clin. Sc., 1953, **12**, 307.
15. Myers, J. D., The hepatic blood flow and splanchnic oxygen consumption of man—their estimation from urea production or bromsulphalein excretion during catheterization of the hepatic veins. J. Clin. Invest., 1947, **26**, 1130.
16. Bondy, P. K., James, D. F., and Farrar, B. W., Studies of the role of the liver in human carbohydrate metabolism by the venous catheter technic. I. Normal subjects under fasting conditions and following the injection of glucose. J. Clin. Invest., 1949, **28**, 238.
17. Bollman, J. L., Khattab, M., Thors, R., and Grindlay, J. H., Experimentally produced alterations of hepatic blood flow. Arch. Surg., 1953, **66**, 562.
18. Neumayr, A., Parzer, O., and Vetter, H., Zur Problematik der Bromsulphalein-Clearance als Leberfunktionsprüfung. Deutsche Med. Wchnschr., 1954, **79**, 1039.
19. Berman, J. K., and Hull, J. E., Circulation in the normal and cirrhotic liver. Ann. Surg., 1953, **137**, 424.
20. Zilversmit, D. B., Boyd, G. A., and Brucer, M., The effect of particle size on blood clearance and tissue distribution of radioactive gold colloids. J. Lab. & Clin. Med., 1952, **40**, 255.
21. Fellinger, K., and Vetter, H., Radiogoldtherapie der leukaemischen Erkrankungen. Symposium on clinical applications of radioactive isotopes, Bad Gastein, January 1954, Strahlenther., In press.
22. Veall, N., and Vetter, H., An apparatus for the rapid estimation of tracer quantities of radioactive isotopes in excreta. Brit. J. Radiol., 1952, **25**, 85.
23. Veall, N., and Baptista, A. M., A multi-tube gamma counting apparatus for small samples. Brit. J. Radiol., 1954, **27**, 198.

AMINO ACID METABOLISM IN INFECTIOUS HEPATITIS[1]

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Although Mann (1) clearly demonstrated the key role of the liver in the deamination process nearly three decades ago, it is only since the development of such techniques as the quantitative measurements of free alpha amino acids in the plasma and urine, and their separation by means of either paper and starch column chromatography or microbiological assay that more definitive studies on the metabolism of amino acids in diseases of the liver can be carried out.

Using microbiological methods, Frankl, Martin, and Dunn (2) first demonstrated considerable variations in the excretion of cystine, tryptophane, and histidine in patients with various types of liver disease. Dunn, Akawaie, Yeh, and Martin estimated that approximately 20 per cent of the patients with liver disease showed increased excretion of amino acids (3). In studies on patients with cirrhosis of the liver, Gabuzda, Eckhardt, and Davidson (4) found only minor disturbances of urine amino acid excretion, the most constant abnormality consisting of increased excretion of methionine and tryptophane, and a decreased excretion of isoleucine. Kinsell, Harper, Barton, Hutchin, and Hess (5) have reported an impaired methionine tolerance in patients with liver disease as compared with normal controls, but Wheeler and György (6) were unable to confirm this.

More recently, Dent and Walshe (7) and Walshe (8, 9) have suggested that because of the enormous reserve of the liver, a study of the total amino nitrogen concentration would not reveal any significant abnormality until the liver is extensively damaged. Using paper partition chromatography techniques, they found gross aminoaciduria in patients with acute yellow atrophy of the liver and hepatic coma, but only minor changes in patients with hepatitis and cirrhosis.

The present study was undertaken to study the changes in amino acid metabolism in infectious hepatitis by: 1) Measuring simultaneously the total alpha amino nitrogen in the plasma and urine, and 2) analyzing the changes of amino acid pattern in the urine by means of paper partition chromatography.

MATERIALS AND METHODS

A total of 18 children with acute infectious hepatitis was studied. The diagnosis was made in every case on the basis of malaise, anorexia, vomiting, fever, abdominal pain, dark urine, light stools, icterus of the skin and sclerae, and an enlarged and tender liver. Liver function tests including cephalin-cholesterol flocculation (10), thymol turbidity (11), thymol flocculation (12), one minute and total serum bilirubin (13, 14) were positive in each patient when first seen. In addition to these tests, increased bile and urobilinogen were demonstrated in the urine and infectious mononucleosis was ruled out by blood smears and heterophil-antibody agglutination. None of the patients had received injections or transfusions within six months prior to the onset of symptoms and contact with infectious hepatitis through the family or school was established in about a third of the cases. Although most of the children were moderately ill during the acute phase of the disease, none developed coma, and all recovered uneventfully.

Since Eckhardt and Davidson (15) and Silber, Seeler, and Howe (16) have previously shown that the urinary excretion of amino acids was only slightly altered by marked changes in protein intake, the patients were placed on a regular diet without restriction during the periods of study. Similarly, although no attempt was made to regulate fluid intake, except in one instance, the 24-hour urinary output was about average for the age and weight of the children studied.

The 24-hour urine collections were preserved with thymol and refrigerated. The urine alpha amino nitrogen was determined by the gasometric ninhydrin-CO_2 method by Van Slyke, MacFadyen, and Hamilton (17). The blood for plasma alpha amino nitrogen was taken two to three hours after the previous meal and determined by the gasometric ninhydrin-CO_2 method of Hamilton and Van Slyke (18).

The amino acids in the 24-hour urine collections were separated by the two dimensional paper partition chromatography using phenol and butanol-acetic acid as

[1] Aided by a grant from the Playtex Park Research Institute.

solvents as described by Consden, Gordon, and Martin (19), and modified by Dent (20, 21). The urine was desalted and concentrated to one-fourth the original volume and 15 μl. of this was analyzed. None of the specimens was hydrolyzed. Each of the amino acids was identified by superimposing with known amino acids and by the use of specific reagents when available (22). The results were tabulated semi-quantitatively by grading against an arbitrary standard, judged under constant lighting conditions in strengths from 1 to 10 units (8).

RESULTS

Plasma and Urine Alpha Amino Nitrogen Measurements

In normal controls

The plasma alpha amino nitrogen was determined in 16 normal children as shown in Table I.

TABLE I

Plasma alpha amino nitrogen levels in normal children

No. subjects:	16
Age of subjects:	3 wks.–8 yrs.
Plasma: *mgm./100 ml.*	
Range:	1.8–5.0
Mean:	3.4
S.D.:	0.9

The values ranged from 1.8 mgm. per cent to 5.0 mgm. per cent with a mean of 3.5 and a S.D. ± 1.0 mgm. per cent. These values are in close agreement with the normal range described by Hamilton and Van Slyke (18) and do not appear to vary with the sex or age of the subject tested.

The urine alpha amino nitrogen was determined in 24-hour collections from 17 children as shown in Table II. The values in the children varied from 0.69 to 1.01 mgm. per pound body weight per 24 hours with a mean of 0.85 and a S.D. ± 0.10 mgm. This is in keeping with the range of approximately 1 mgm. per pound body weight per 24 hours reported by Childs (23) and 2 mgm. per kilogram body weight per 24 hours reported by Jonxis (24) for normal children.

TABLE II

Twenty-four-hour urine alpha amino nitrogen excretion in normal children

No. subjects:	17
Age of subjects:	8 wks.–15 yrs.
Wt. of subjects:	11 lbs.–113 lbs.
Urine volume:	95 cc.–1,960 cc.
Urine: *mgm./lb./24 hr.*	
Range:	0.69–1.01
Mean:	0.85
S.D.:	0.09

In patients with acute infectious hepatitis

The plasma and urine alpha amino nitrogen values during the acute phase of infectious hepatitis are shown in Table III. One-third of the patients showed a moderate increase of urinary alpha amino nitrogen with an output of 50 per cent or more above that of the controls. Another third of the patients showed borderline changes with an output which varied between 20 and 50 per cent above normal. These changes are not marked, but are sufficiently above the normal range to be noteworthy. The remaining third of the patients showed normal urinary output of alpha amino nitrogen.

Simultaneous determinations of plasma alpha amino nitrogen also showed borderline increases. In the group of patients with moderate amino-aciduria, all five of the cases had values of more than 5.0 mgm. per cent and three of these above 5.5 mgm. per cent. In the borderline group, three of the six cases showed values of more than 5.0 mgm. per cent, but all were below 5.5 mgm. per cent. In the group exhibiting no amino-aciduria, the plasma alpha amino nitrogen in all five of the patients tested was found to be within the normal range. These determinations appear to be relatively unaffected by the sex, age, body weight, or urinary volume of the patients. Furthermore, the amino-aciduria did not correlate with the severity of disease clinically nor with the interval between the onset of symptoms and collection of samples.

In patients following recovery from infectious hepatitis

All six of the patients with moderate amino-aciduria, and three of the six patients with borderline amino-aciduria were restudied 5 to 18 weeks following the onset of symptoms. By then, none of them showed any signs of disease and the liver function tests had all returned to normal values. It can be seen from Table III that in the moderate amino-aciduria group, the plasma alpha amino nitrogen was within the normal range in all except Case 6, where some increase persisted. Simi-

TABLE III

*Plasma and urine alpha amino nitrogen determinations in patients with infectious hepatitis**

Sex	Age (years)	Wt. (lbs.)	Plasma amino N (mgm./100 cc.)		Urine amino nitrogen (Volume cc.)		(mgm./24 hrs.)		(mgm./lb./24 hrs.)	
			Acute phase	Follow-up	Acute phase	Follow-up	Acute phase	Follow-up	Acute phase	Follow-up
Moderate amino aciduria (Increase of more than 50%)										
M	7	58	5.2	3.9	1,660	930	120.0	58.0	2.07	1.00
M	10	75	5.2	3.4	2,900	1,900	145.5	76.0	1.94	1.00
M	9	55	5.7	2.5	620	960	95.4	38.2	1.73	0.70
M	10	78	—	4.3	600	950	123.6	73.3	1.58	0.94
F	2	27	6.0	2.9	490	550	37.7	35.0	1.40	1.29
M	10½	75	5.6	2.7	770	850	103.0	71.2	1.38	0.95
Average			5.5	3.3					1.70	0.98
Borderline amino aciduria (20% to 50% above normal)										
M	7	86	2.7	3.9	680	400	103.0	60.0	1.20	0.70
M	7	42	5.3	—	480	—	48.0	—	1.15	—
F	6	46	3.4	3.8	680	440	52.3	35.4	1.14	0.77
M	10	68	5.3	—	580	—	72.5	—	1.07	—
F	9	64	2.5	3.6	520	290	67.6	30.0†	1.05	0.47†
M	10	72	5.1	—	740	—	78.0	—	1.04	—
Average			2.9	3.8					1.11	0.65
No amino aciduria (Normal values)										
F	8	58	—	—	660	—	52.7	—	0.91	—
F	10	70	2.6	—	610	—	61.0	—	0.87	—
M	8	60	3.4	—	540	—	48.0	—	0.80	—
M	7	54	2.2	—	880	—	41.4	—	0.77	—
M	6	48	2.8	—	520	—	36.4	—	0.76	—
F	6	48	3.3	—	620	—	32.9	—	0.69	—
Average			2.9						0.80	

* Values include those during the acute phase of the disease and in follow-up studies after convalescence.
† Part of specimen accidentally lost.

larly, in the group with borderline amino-aciduria, plasma and urine alpha amino nitrogen was within normal limits in all three of the patients restudied.

Amino Acid Pattern by Paper Partition Chromatography

In normal controls

The distribution of specific amino acids analyzed in 24-hour collections of urine from six adults and nine children, who acted as normal controls is shown in Table IV. Alanine, glutamine, glycine, histidine, and serine were prominent in most cases. This agrees with the studies in normal individuals carried out by Woolf (25) and Dent (26) except for a greater prominence of taurine in the latter's cases and a more frequent appearance of histidine in the present group. These differences could be attributed to: 1) Loss of amino acids through desalting; 2) differences in the solvents used; and 3) studies based on 24-hour urine collections instead of single morning specimens.

In patients with acute infectious hepatitis

The distribution of amino acids in the 18 patients with infectious hepatitis is shown in Table V. Of the amino acids usually present in the urine, alanine, glycine, glutamine, and serine showed increases. In addition, many of the essential amino acids were seen in the urine of a number of these patients. These included moderate increases of isoleucine/leucine, lysine, and

solvents as described by Consden, Gordon, and Martin (19), and modified by Dent (20, 21). The urine was desalted and concentrated to one-fourth the original volume and 15 µl. of this was analyzed. None of the specimens was hydrolyzed. Each of the amino acids was identified by superimposing with known amino acids and by the use of specific reagents when available (22). The results were tabulated semi-quantitatively by grading against an arbitrary standard, judged under constant lighting conditions in strengths from 1 to 10 units (8).

RESULTS

Plasma and Urine Alpha Amino Nitrogen Measurements

In normal controls

The plasma alpha amino nitrogen was determined in 16 normal children as shown in Table I.

TABLE I

Plasma alpha amino nitrogen levels in normal children

No. subjects:	16
Age of subjects:	3 wks.–8 yrs.
Plasma: *mgm./100 ml.*	
Range:	1.8–5.0
Mean:	3.4
S.D.:	0.9

The values ranged from 1.8 mgm. per cent to 5.0 mgm. per cent with a mean of 3.5 and a S.D. ± 1.0 mgm. per cent. These values are in close agreement with the normal range described by Hamilton and Van Slyke (18) and do not appear to vary with the sex or age of the subject tested.

The urine alpha amino nitrogen was determined in 24-hour collections from 17 children as shown in Table II. The values in the children varied from 0.69 to 1.01 mgm. per pound body weight per 24 hours with a mean of 0.85 and a S.D. ± 0.10 mgm. This is in keeping with the range of approximately 1 mgm. per pound body weight per 24 hours reported by Childs (23) and 2 mgm. per kilogram body weight per 24 hours reported by Jonxis (24) for normal children.

TABLE II

Twenty-four-hour urine alpha amino nitrogen excretion in normal children

No. subjects:	17
Age of subjects:	8 wks.–15 yrs.
Wt. of subjects:	11 lbs.–113 lbs.
Urine volume:	95 cc.–1,960 cc.
Urine: *mgm./lb./24 hr.*	
Range:	0.69–1.01
Mean:	0.85
S.D.:	0.09

In patients with acute infectious hepatitis

The plasma and urine alpha amino nitrogen values during the acute phase of infectious hepatitis are shown in Table III. One-third of the patients showed a moderate increase of urinary alpha amino nitrogen with an output of 50 per cent or more above that of the controls. Another third of the patients showed borderline changes with an output which varied between 20 and 50 per cent above normal. These changes are not marked, but are sufficiently above the normal range to be noteworthy. The remaining third of the patients showed normal urinary output of alpha amino nitrogen.

Simultaneous determinations of plasma alpha amino nitrogen also showed borderline increases. In the group of patients with moderate amino-aciduria, all five of the cases had values of more than 5.0 mgm. per cent and three of these above 5.5 mgm. per cent. In the borderline group, three of the six cases showed values of more than 5.0 mgm. per cent, but all were below 5.5 mgm. per cent. In the group exhibiting no amino-aciduria, the plasma alpha amino nitrogen in all five of the patients tested was found to be within the normal range. These determinations appear to be relatively unaffected by the sex, age, body weight, or urinary volume of the patients. Furthermore, the amino-aciduria did not correlate with the severity of disease clinically nor with the interval between the onset of symptoms and collection of samples.

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All six of the patients with moderate amino-aciduria, and three of the six patients with borderline amino-aciduria were restudied 5 to 18 weeks following the onset of symptoms. By then, none of them showed any signs of disease and the liver function tests had all returned to normal values. It can be seen from Table III that in the moderate amino-aciduria group, the plasma alpha amino nitrogen was within the normal range in all except Case 6, where some increase persisted. Simi-

TABLE III

*Plasma and urine alpha amino nitrogen determinations in patients with infectious hepatitis**

Sex	Age (*years*)	Wt. (*lbs.*)	Plasma amino N (*mgm./100 cc.*)		Urine amino nitrogen (*Volume cc.*)		(*mgm./24 hrs.*)		(*mgm./lb./24 hrs.*)	
			Acute phase	Follow-up	Acute phase	Follow-up	Acute phase	Follow-up	Acute phase	Follow-up
Moderate amino aciduria (Increase of more than 50%)										
M	7	58	5.2	3.9	1,660	930	120.0	58.0	2.07	1.00
M	10	75	5.2	3.4	2,900	1,900	145.5	76.0	1.94	1.00
M	9	55	5.7	2.5	620	960	95.4	38.2	1.73	0.70
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F	2	27	6.0	2.9	490	550	37.7	35.0	1.40	1.29
M	10½	75	5.6	2.7	770	850	103.0	71.2	1.38	0.95
Average			5.5	3.3					1.70	0.98
Borderline amino aciduria (20% to 50% above normal)										
M	7	86	2.7	3.9	680	400	103.0	60.0	1.20	0.70
M	7	42	5.3	—	480	—	48.0	—	1.15	—
F	6	46	3.4	3.8	680	440	52.3	35.4	1.14	0.77
M	10	68	5.3	—	580	—	72.5	—	1.07	—
F	9	64	2.5	3.6	520	290	67.6	30.0†	1.05	0.47†
M	10	72	5.1	—	740	—	78.0	—	1.04	—
Average			2.9	3.8					1.11	0.65
No amino aciduria (Normal values)										
F	8	58	—	—	660	—	52.7	—	0.91	—
F	10	70	2.6	—	610	—	61.0	—	0.87	—
M	8	60	3.4	—	540	—	48.0	—	0.80	—
M	7	54	2.2	—	880	—	41.4	—	0.77	—
M	6	48	2.8	—	520	—	36.4	—	0.76	—
F	6	48	3.3	—	620	—	32.9	—	0.69	—
Average			2.9						0.80	

* Values include those during the acute phase of the disease and in follow-up studies after convalescence.
† Part of specimen accidentally lost.

larly, in the group with borderline amino-aciduria, plasma and urine alpha amino nitrogen was within normal limits in all three of the patients restudied.

Amino Acid Pattern by Paper Partition Chromatography

In normal controls

The distribution of specific amino acids analyzed in 24-hour collections of urine from six adults and nine children, who acted as normal controls is shown in Table IV. Alanine, glutamine, glycine, histidine, and serine were prominent in most cases. This agrees with the studies in normal individuals carried out by Woolf (25) and Dent (26) except for a greater prominence of taurine in the latter's cases and a more frequent appearance of histidine in the present group. These differences could be attributed to: 1) Loss of amino acids through desalting; 2) differences in the solvents used; and 3) studies based on 24-hour urine collections instead of single morning specimens.

In patients with acute infectious hepatitis

The distribution of amino acids in the 18 patients with infectious hepatitis is shown in Table V. Of the amino acids usually present in the urine, alanine, glycine, glutamine, and serine showed increases. In addition, many of the essential amino acids were seen in the urine of a number of these patients. These included moderate increases of isoleucine/leucine, lysine, and

TABLE IV

*Distribution of amino acids by paper partition chromatography in the urine of normal controls**

AMINO ACIDS	Rf Phenol	Rf But-Acet	ADULTS 22M	22M	28M	26M	40F	22M	CHILDREN 6M	12F	3M	6M	3M	7M	3M	8M	6F	MEAN
ALANINE	.6½	.38	2		2	4	3	2	1	1	2	2	2	2		2	2	1.7
ARGININE	.85	.23								1					2			0.2
ASPARAGINE	.49	.30																
ASPARTIC ACID	.20	.23																
CYSTINE	.42	.17						4								4		0.5
GLUTAMIC ACID	.30	.34						4										0.2
GLUTAMINE	.57	.22	2	2	3	2			2	2	2					2	2	1.3
GLYCINE	.45	.36	5	4	5	5	4	3	4	4	4	4	4	4	4	3	3	4.0
HISTIDINE	.74	.21	2	1	4	2	3	3	3	3	4		4		4		2	2.3
HYDROXY-L-PROLINE	.51	.36																
ISOLEUCINE/LEUCINE	.79	.64																
LYSINE	.72	.15																
METHIONINE	.77	.57																
METHYLHISTIDINE	—	—																
PHENYLALANINE	.91	.60																
PROLINE	.40	.42																
SERINE	.41	.25	2	2	2		1		2		2		3			4	2	1.3
TAURINE	.47	.25																
THREONINE	.51	.30			1									2		2		0.3
TRYPTOPHANE	.76	.59										2						0.1
TYROSINE	.55	.59			2						1						2	0.3
VALINE	.81	.52	1		2		2										2	0.5
L AMINO ISO-BUTYRIC	.66	.40	1						2	3	1	3	1				2	0.8
Total No. Amino Acids			6	4	8	4	5	5	6	7	6	4	5	3	4	6	7	
Total No. Units			15	9	21	13	13	16	14	14	16	11	14	8	10	17	17	
			Mean 14 units															

*Average value for entire group to nearest 0.1 unit.

* Age and sex of subjects given at top of each column.

methionine, and slight increases of arginine, phenylalanine, threonine, and valine. A slight increase of tyrosine was also noted. This is in general agreement with the findings of Walshe (9) except for the absence of taurine and only a slight increase of cystine. The authors have been unable properly to identify *B*-amino-iso-butyric acid (27) and hence this substance cannot be evaluated in the present study.

There is a close correlation between the amino acid pattern and the total alpha amino nitrogen in the urine. In the group with moderate amino-aciduria, the excretion averaged 26 units. All except Case 2 showed higher levels than that of the controls. In that patient, the urinary volume was so large that the concentration of the amino acids was greatly diluted and hence they were not so prominent. In the group with borderline amino-aciduria, the amino acid excretion averaged 21 units, which is still higher than in the controls. In the so-called normal group, the amino acid excretion averaged 17 units, which represents only a slight increase over the controls. In individual patients, several of the amino acids were much more prominent than among the controls.

In patients following recovery from acute hepatitis

The distribution of amino acids in the nine patients following recovery from infectious hepatitis is shown in Table VI. Except for a continued increase of glutamine in most of the patients, the amino acids appear to have returned to normal levels.

TABLE V

Distribution of amino acids by paper partition chromatography in the urine of patients with acute infectious hepatitis

AMINO ACIDS	CASES																		MEAN*
	MODERATE AMINO-ACIDURIA						BORDERLINE AMINO-ACIDURIA						NO AMINO-ACIDURIA						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
ALANINE	4	2	2	3	2	4	4	2	4	4	2	3	3	3	2	5	2	6	3.2
ARGININE			2	2				2		1		1	1						0.6
ASPARAGINE																			
ASPARTIC ACID													2			1			0.2
CYSTINE			2	4	4							2							0.7
GLUTAMIC ACID									1	1						2			0.2
GLUTAMINE	4	2	4	4	4	4	4	4		4	2		1		2		4		2.4
GLYCINE	6	5	6	7	6	6	6	6	7	6	6	6	4	6	4	3	4	4	5.4
HISTIDINE	4		4		2	4			1		4		1	3	4			2	1.6
HYDROXY-L-PROLINE																			
ISO LEUCINE/LEUCINE	4		2	2		2	2			2	1	1	2	1	2				1.2
LYSINE				3	2							4							0.6
METHIONINE			1	1	2					1	1	2	3		2				0.7
METHYLHISTIDINE																			
PHENYLALANINE	2	1				2		2										1	0.4
PROLINE																			
SERINE	6	1	6	3	2				6		2		2	2	2		2		1.9
TAURINE																			
THREONINE						1			4		2	1							0.6
TRYPTOPHANE	2					1							2						0.3
TYROSINE			1	3	1		2				2	1			1			1	0.7
VALINE			2					4		2	1				1			2	0.8
L AMINO ISO-BUTYRIC		1							1	1	1			1		4	2	2	0.7
Total No. Amino Acids	8	6	11	10	9	8	5	6	7	9	11	9	10	6	9	5	5	7	
Total No. Units	32	12	32	32	25	24	18	20	24	22	24	21	21	16	20	15	14	18	
	Mean 26 units						Mean 21 units						Mean 17 units						

TABLE VI

Distribution of amino acids by paper partition chromatography in the urine following recovery from infectious hepatitis

	CASE NO.									
	1	2	3	4	5	6	7	9	11	Mean
ALANINE	3	2		2	2	3	2	3	3	2.2
ARGININE							1			0.1
ASPARAGINE										
ASPARTIC ACID										
CYSTINE										
GLUTAMIC ACID										
GLUTAMINE	4	1	1	2	3	2	2	1	3	2.1
GLYCINE	6	3	2	4	6	5	5	6	5	4.6
HISTIDINE										
HYDROXY-L-PROLINE			2		1		2	2	1	0.9
ISO LEUCINE|LEUCINE										
LYSINE						2		1		0.3
METHIONINE										
METHYLHISTIDINE										
PHENYLALANINE										
PROLINE										
SERINE	3				2	2		1		0.9
TAURINE										
THREONINE	1									0.1
TRYPTOPHANE	1				1	1	1			0.5
TYROSINE					2	2	1	1	1	0.8
VALINE										
L AMINO ISO-BUTYRIC	2			1		1			1	0.5
Total No. Amino Acids	7	3	3	4	7	8	7	7	6	
Total No. Units	20	6	5	9	17	18	14	15	14	
	Mean 13 units									

DISCUSSION

At least two different mechanisms for amino-aciduria have been postulated: 1) It occurs as a result of increased amino acid levels in the plasma which exceeds the normal renal threshold and results in an overflow into the urine. The amino-aciduria associated with progressive muscular dystrophy, cystinosis, phenylketonuria, idiopathic hypoproteinemia with edema in infancy, and severe liver disease has been attributed to this type of mechanism; 2) it can also occur as a result of dysfunction of the renal tubules, which causes poor reabsorption and increased excretion of amino acids. This can be an inherent congenital defect as in deToni-Fanconi syndrome, cystinuria, Wilson's Disease, and rickets, or a transient defect as a result of toxic action noted in lead, uranium, nitrobenzene, "lysol" poisoning, and the ingestion of galactose in galactosemia.

Bollman, Mann, and Magath (28) have shown that the removal of the liver in dogs results in complete cessation of deamination. This was demonstrated by the recovery of amino acids in the blood, urine, and tissues in such animals approximately equal to the anticipated formation of urea had the animals been normal. If amino acids were injected into these animals, the entire amount of amino acid nitrogen could be recovered unchanged many hours after administration. Although infectious hepatitis has generally been regarded as a mild disease, the present data suggest that it may cause sufficient damage to interfere with deamination. The amino acid disturbances follow essentially the same, though a much milder pattern, than that following hepatectomy. If instead of measuring only the total alpha amino nitrogen, we had measured each of the individual amino acids separately, the changes in some would probably have been more striking since the total alpha amino nitrogen reflects not only the increase in certain of the amino acids, but other amino acids which either remain unchanged or actually are decreased in this disease.

The data also provide an opportunity to speculate on the mechanism for overflow of plasma amino acids into the urine. Silber, Seeler, and Howe (16) have previously shown that rapid infusion of amino acid mixture resulted in a large increase of plasma alpha amino nitrogen, but only moderate or slight increase of urine alpha amino nitrogen. However, since these infusions were run over a relatively short period of time and produced changes in total blood volume, the conditions for study were somewhat different from those in a group of patients with elevations of alpha amino nitrogen due to disease. Although the increase of both plasma and urine alpha amino nitrogen in this study were slight, they tend to confirm Silber's findings, and show that in this type of amino-aciduria, the changes in the urine tend to be slight and subtle, and are quite different from the gross changes which occur when a tubular defect of the kidneys exist.

Although it would be tempting to attribute all of the amino acid changes in infectious hepatitis to failure of deamination, a separate renal tubular defect cannot be completely eliminated on the basis of the present data (29). Pitts (30) has shown that the quantitative characteristics of the reabsorptive processes are different for each of the amino acids and that certain amino acids may interfere with the absorption of others. To rule out definitely a tubular defect, therefore, one must not only have quantitative measurements of each of the amino acids, but also understand their action in combination with others. There is some evidence, however, to show that the clearance of the amino acids in infectious hepatitis may not be abnormal. In the first place, Pitts (31) has shown that with the rise of plasma glycine concentration, the rate of increased reabsorption fails to increase in proportion to the amount filtered and consequently excretion may become appreciable. This appears to occur with glycine, alanine, glutamine, and serine in normal individuals, and could occur with some of the other essential amino acids when their concentrations are increased in the plasma. Secondly, Walshe (9) has shown that in severe liver disease, methionine is greatly increased, alanine, glutamine, serine, arginine, cystine, tyrosine, lysine, and phenylalanine are moderately increased. In the present study, the urinary excretion of alanine, glycine, glutamine, and leucine/isoleucine was markedly increased, lysine, methionine, and serine were moderately increased, and arginine, phenylalanine, threonine, valine, and tyrosine were slightly increased. In load experiments in normal children, Jonxis (32) has found

that serine, threonine, glycine, alanine, and histidine are not as well reabsorbed as arginine, tryptophane, tyrosine, and phenylalanine are. The remaining amino acids lie between the two groups. Taking this into consideration, it would appear that except for cystine, most of the increases of the amino acids in the urine could be accounted for by the changes in the plasma in liver disease.

During recent years, high protein diets and supplements such as choline and methionine have been recommended as therapy for acute hepatitis. The present data would seem to suggest that such therapy is unimportant during the acute phase of the disease since there is no actual shortage of amino acids, but only an inability of the liver to deaminate them. Increasing the load of the amino acids either by mouth or parentally will only result in increased excretion.

SUMMARY

1. The changes in amino acid metabolism have been studied in 19 children with infectious hepatitis.

2. Measurements of the total urine alpha amino nitrogen by the gasometric ninhydrin-CO_2 method showed that one-third of the cases had a moderate amino-aciduria, another third had a borderline amino-aciduria, and the remaining third showed normal amino acid excretion.

3. Plasma alpha amino nitrogen studied by the same method was high in all of the patients with moderate amino-aciduria and in some of those with borderline amino-aciduria. All were within normal limits in those without increased amino acid excretion.

4. Both the urine and plasma alpha amino nitrogen returned to normal levels upon recovery.

5. Analysis of the specific amino acids by paper partition chromatography showed a generalized amino-aciduria in all cases with most of the increase among essential amino acids.

6. These changes in amino acid metabolism can be attributed to disturbances in deamination. Further investigation is needed to determine whether or not an additional renal defect exists.

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REFERENCES

1. Mann, F. C., The effects of complete and partial removal of the liver. Medicine, 1927, **6**, 419.
2. Frankl, W., Martin, H., and Dunn, M. S., The apparent concentration of free tryptophan, histidine, and cystine in pathological human urine measured microbiologically. Arch. Biochem., 1947, **13**, 103.
3. Dunn, M. S., Akawaie, S., Yeh, H. L., and Martin, H., Urinary excretion of amino acids in liver disease. J. Clin. Invest., 1950, **29**, 302.
4. Gabuzda, G. J., Jr., Eckhardt, R. D., and Davidson, C. S., Urinary excretion of amino acids in patients with cirrhosis of the liver and in normal adults. J. Clin. Invest., 1952, **31**, 1015.
5. Kinsell, L. W., Harper, H. A., Barton, H. C., Hutchin, M. E., and Hess, J. R., Studies in methionine and sulfur metabolism. I. The fate of intravenously administered methionine in normal individuals and in patients with liver damage. J. Clin. Invest., 1948, **27**, 677.
6. Wheeler, J. E., and György, P., Studies of urinary excretion of methionine by normals and by patients having liver disease. Am. J. M. Sc., 1948, **215**, 267.
7. Dent, C. E., and Walshe, J. M., Amino-acid metabolism in liver disease. In a Ciba Foundation Symposium *on* Liver Disease, S. Sherlock and G. E. W. Wolstenholme, Eds., Philadelphia, Blakiston, 1951, p. 22.
8. Walshe, J. M., Observations on the symptomatology and pathogenesis of hepatic coma. Quart. J. Med., 1951, **20**, 421.
9. Walshe, J. M., Disturbances of aminoacid metabolism following liver injury. Quart. J. Med., 1953, **22**, 483.
10. Hanger, F. M., Serological differentiation of obstructive from hepatogenous jaundice by flocculation of cephalin-cholesterol emulsions. J. Clin. Invest., 1939, **18**, 261.
11. Maclagan, N. F., Thymol turbidity test: A new indicator of liver dysfunction. Nature, 1944, **154**, 670.
12. Neefe, J. R., Results of hepatic tests in chronic hepatitis without jaundice; correlation with clinical course and liver biopsy findings. Gastroenterology, 1946, **7**, 1.
13. Malloy, H. T., and Evelyn, K. A., The determination of bilirubin with the photoelectric colorimeter. J. Biol. Chem., 1937, **119**, 481.
14. Ducci, H., and Watson, C. J., The quantitative determination of serum bilirubin with special reference to the prompt-reacting and chloroform-soluble types. J. Lab. & Clin. Med., 1945, **30**, 293.
15. Eckhardt, R. D., and Davidson, C. S., Urinary excretion of amino acids by a normal adult receiving

diets of varied protein content. J. Biol. Chem., 1949, **177**, 687.

16. Silber, R. H., Seeler, A. O., and Howe, E. E., Urinary excretion of α-amino nitrogen following intravenous administration of amino acid mixtures. J. Biol. Chem., 1946, **164**, 639.
17. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., The gasometric determination of amino acids in urine by the ninhydrin-carbon dioxide method. J. Biol. Chem., 1943, **150**, 251.
18. Hamilton, P. B., and Van Slyke, D. D., The gasometric determination of free amino acids in blood filtrates by the ninhydrin-carbon dioxide method. J. Biol. Chem., 1943, **150**, 231.
19. Consden, R., Gordon, A. H., and Martin, A. J. P., Qualitative analysis of proteins: A partition chromatographic method using paper. Biochem. J., 1944, **38**, 224.
20. Dent, C. E., Detection of amino-acids in urine and other fluids. Lancet, 1946, **2**, 637.
21. Dent, C. E., Paper chromatography and medicine *in* Recent Advances in Clinical Pathology, Second edition, S. C. Dyke, Ed., Philadelphia, the Blakiston Co., 1952, p. 238.
22. Block, R. J., LeStrange, R., and Zweig, G., Paper chromatography, 1952, a laboratory manual. New York, Academic Press, 1952.
23. Childs, B., Urinary excretion of free alpha-amino acid nitrogen by normal infants and children. Proc. Soc. Exper. Biol. & Med., 1952, **81**, 225
24. Jonxis, J. H. P., Personal communication.
25. Woolf, L. I., Paper chromatography. Great Ormond Street Journal, 1951, No. **2**, 61.
26. Dent, C. E., Proc. 1st International Congress of Clinical Pathology. London, 1951, p. 42.
27. Crumpler, H. R., Dent, C. E., Harris, H., and Westall, R. G., *B*-aminoisobutyric acid: A new amino-acid obtained from human urine. Nature, 1951, **167**, 307.
28. Bollman, J. L., Mann, F. C., and Magath, T. B., Studies on the physiology of the liver. XV. Effect of total removal of the liver on deamination. Am. J. Physiol., 1926, **78**, 258.
29. Smith, H. W., The Kidney, Structure and Function in Health and Disease. New York, Oxford Univ. Press, 1951, p. 104.
30. Pitts, R. F., A renal reabsorptive mechanism in the dog common to glycin and creatine. Am. J. Physiol., 1943, **140**, 156.
31. Pitts, R. F., A comparison of the renal reabsorptive processes for several amino acids. Am. J. Physiol., 1943, **140**, 535.
32. Jonxis, J. H. P., Unpublished data.

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THE INFLUENCE OF ANTIBIOTICS ON THE ORIGIN OF SMALL COLONIES (G VARIANTS) OF *MICROCOCCUS PYOGENES* VAR. *AUREUS*[1]

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In attempting to select out penicillin-resistant colonies of staphylococcus from sensitive cultures by the method of Kirby (1), small G colonies were obtained. When isolated on plates of agar, the colonies in some instances appeared so minute, that growth was detected only with the aid of magnification. A study of this phenomenon was pursued further with many cultures of staphylococcus and with different antibiotics, and minute colonies were again isolated. The small colonies reverted to the normal larger colonies when cultured in the absence of antibiotics. Since the minute colonies originated under the influence of antibiotics commonly used in the therapy of staphylococcal diseases, the possibility presented itself that these colonies might also appear in patients while undergoing treatment with antibiotics.

This report is concerned first, with a brief summary of the literature on G colonies; second, experiments relating to the *in vitro* conditions under which the minute colonies appeared with the different antibiotics; third, changes in virulence and resistance to the antibiotics displayed by the parent colonies, small colonies, and the reverted large colonies; fourth, the sensitivity to bacteriophage of parent colonies, G colonies and reverted colonies; and, lastly, the isolation of minute colonies from human patients with staphylococcal disease.

REVIEW OF LITERATURE

Small colony variants have been described for many species of bacteria. Most of the variants reported by investigators were obtained from cultures submitted to unfavorable metabolic influences, such as chemicals, antibiotics, and aging. In some instances, isolation was from patients following treatment with antibiotics. Interest in small colonies was stimulated in 1931 by Hadley, Delves, and Klimek (2), who isolated very small colonies of *Shigella* from broth cultures containing lithium chloride, and reported that they passed through bacteriological filters, and suggested that they were gonidial mutants and part of a normal life cycle. Prior to their report, other workers (3–8) had noted similar types of colonies.

The method of Hadley, which incorporates selective inhibitory substances in bacteriological media, has been widely used for the isolation of G colonies from cultures of different bacterial species. These substances include lithium chloride, barium chloride, 2-methyl-1,4-naphthoquinone, and sterile filtrates of bacterial cultures (9–23). Appearance of G colonies in cultures of filtrates of "transitional" colonies of *Salmonella*, which appeared on MacConkey agar, has also been reported (24). Small colonies of the G type have been isolated, without the aid of added inhibitory substances, from old cultures of bacteria and yeasts (12, 25–37).

The selection of G colonies with antibiotics *in vitro* was first described by Schnitzer, Camagni, and Buck (20) when they observed small colonies in areas where growth of staphylococci had been inhibited by a crude extract of *Penicillium notatum*. These minute colonies reverted to normal colonial forms when cultivated in the absence of the antibiotic. Similar observations were reported by others (38, 39) who used more purified preparations of penicillin. Barbour (40) noted that streptomycin caused the appearance of G forms during the development of resistance in five strains of *Staphylococcus pyogenes*.

The primary isolation of G cultures from animal or human sources is of considerable clinical interest, for little is known concerning the possible role of such bacterial variants in disease.

[1] Presented at the Forty-fifth Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 4, 1953.

Edwards (41) encountered "dwarf" colonies of *Shigella equerulis*, an organism associated with a disease of horses, in 25 per cent of post mortem examinations. Bliss and Long (42) observed almost invisible colonies of hemolytic streptococci which were isolated from throats of patients with upper respiratory infections. Small colonies of gonococci were isolated by Morton and Shoemaker (43) from 3 of 135 cases of gonorrhea. It was not known whether these three cases had received treatment. Morris, Sellers, and Brown (44) isolated from blood, feces, urine, and sputum small colony variants of *Eberthella typhosa* from 10 of many patients between 1934 and 1940. Hall and Spink (45) isolated streptomycin-resistant and streptomycin-dependent small colonies of *Brucella abortus* from a patient with subacute bacterial endocarditis following treatment with streptomycin. Voureka (46) obtained small colonies from urine of patients with urinary-tract infections caused by *Pseudomonas* and coliform organisms following treatment with chloramphenicol. Very small colonies of staphylococcus have been isolated in pure culture from closed abscesses of three patients. In the case reported by Hale (47) small colonies were isolated from the abscess, as well as from the patient's nose. One of two patients reported by Sherris (48) had never received antibiotics; the other had been treated with penicillin.

The general characteristics of G colonies, which have been described for many species of bacteria are similar in most instances but there have been some reports at variance with each other. The reported characteristics may be summarized briefly as follows: Colonies are translucent, sometimes opaque, do not produce pigment and vary in size from 0.02 to 1 mm. (9, 12, 18, 24, 27, 40). Broth cultures exhibit slight turbidity. Stained specimens have revealed microorganisms which were described as normal and uniform, or irregular and small. The germination period has been described as 1 hour and 45 minutes to 3 hours and 50 minutes for the G organisms, as compared to 36 minutes for the parent organisms (27). Hemolytic, coagulating and fermentative activity is decreased. The size of the colonies is increased by cultivation in an environment of CO_2 or by the use of thiamine, or dextrose and blood in the media (12, 17, 47). Bacteriophage susceptibility between parent cultures and their G cultures is reported to be different, the same, or decreased in case of the G colony (9, 11, 21, 24, 40). Cross agglutination with antisera produced with the parent cultures and with the cultures of G colonies is reported to occur in most cases (6, 9, 12, 15, 26, 27, 30, 34, 41, 45). However, some investigators (2, 3) report no cross agglutination and others (11, 24) state that antisera prepared against G cultures do not agglutinate parent cultures, whereas antisera prepared against parent cultures agglutinate both parent and G cultures. Most investigators (2, 9, 12, 41) have reported the G cultures to be nonvirulent; others (18, 24, 26, 32) report a decrease in virulence; whereas Hadley and Carapetian (15) found an increase in virulence. Heat resistance of G colonies is increased or decreased (9, 21, 22). The rate of reduction of methylene blue is decreased (27, 31). There is a decreased catophoretic velocity (27, 31). The most controversial property of the G organisms is the question of their passage through bacteriological filters. They have been reported by some (2, 9, 15, 24, 25, 28) to be filterable, but others (12, 14, 16, 21, 26, 27, 30, 31, 33) have not been able to demonstrate this characteristic.

The nature and function of G colonies are not known. Hadley, Delves, and Klimek (2) believed them to be part of a normal life cycle. Youmans (13) considered them to be bacteria with a temporary loss of inactivity of certain enzyme functions and a lowered rate of metabolism, brought on by an unfavorable environment. A similar view was expressed by Weinberg (17), who thought that they were biochemically deficient variants. Lindegren (49) stated that the normal G-type transformation resembled very closely a transgenation (point mutation, gene mutation) which he believed to serve as an adaptive device.

MATERIALS AND METHODS

For the selection of G colonies of staphylococci with antibiotics, a method was employed similar to that used by Kirby (1) in studying the action of penicillin on staphylococci. Large inocula of a broth culture of staphylococci, which had been incubated for 18 hours, were added to each of several Klett-Summerson colorimeter tubes containing 5 ml. of tryptose phosphate broth [2] and

[2] Difco Laboratories, Inc., Detroit, Michigan.

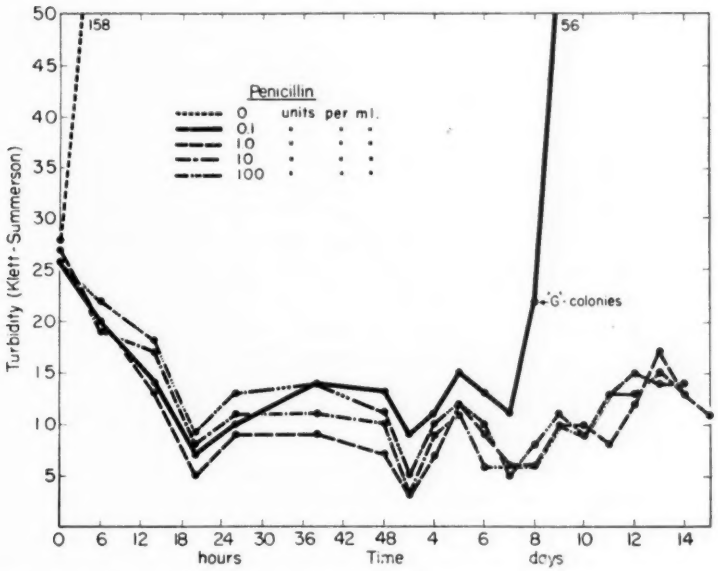

FIG. 1. INFLUENCE OF VARYING CONCENTRATIONS OF PENICILLIN UPON BROTH CULTURES OF STAPHYLOCOCCUS

Lysis of the organisms occurred with all the concentrations, but G colonies appeared only in the tube with lowest concentration of penicillin—0.1 unit per ml.

different concentrations of antibiotics. The antibiotics used were penicillin, streptomycin, chloramphenicol, bacitracin, carbomycin, and oxytetracycline in concentrations of 0.1, 1.0, 10.0, and 100.0 units or micrograms per ml., and erythromycin in concentrations of 0.25, 2.5, 5.0, 10, 20, 40, and 80 micrograms per ml. The bacterial inoculum added to each tube was that amount necessary to produce an increase of 25 units in the turbidity of broth, as measured by a Klett-Summerson photoelectric colorimeter, and varied from 0.3 to 1.0 ml. in all the experiments, but a constant amount was used with each antibiotic. A series of control tubes were prepared to determine the rate of growth in the absence of antibiotics, to indicate the possibility of contamination of uninoculated broth containing antibiotics, and to serve as a control for turbidimetric readings. All tubes were incubated at 37° C. The turbidity of each tube was determined at frequent intervals for 14 days. Every two days a portion of the contents of each tube was subcultured to plates of trypticase soy agar.[3] After incubation the agar plates were examined with the aid of a stereoscopic microscope and selected small colonies were subcultured in broth for 24 hours. Reverted large colonies (RV) were obtained from all G cultures. All cultures were maintained on agar plates in the refrigerator and in broth at minus 10° C.

[3] Baltimore Biological Products Laboratory, Baltimore, Md.

RESULTS

Thirty individual strains of coagulase-positive staphylococci were studied. All strains were susceptible to low concentrations of all the antibiotics. The number of strains tested with each antibiotic was as follows: penicillin, 20; erythromycin, 10; carbomycin, 9; bacitracin, 9; streptomycin, 13; oxytetracycline, 8; and chloramphenicol, 8. Although many small colonies were selected for subculture, the majority yielded large cultures after 24 hours of incubation. However, fifteen stable G cultures were obtained. The number of G cultures produced with each antibiotic was as follows: penicillin, 6; erythromycin, 2; carbomycin, 3; and bacitracin, 4. None was isolated in the presence of streptomycin and chloramphenicol. Color changes which occurred with oxytetracycline precluded adequate determinations of turbidity, and no G colonies were obtained with this antibiotic. In most instances, G colonies were isolated after the parent culture had been lysed by the antibiotic, and were present in only one concentration of the antibiotic.

The pattern of changes of turbidity of the cul-

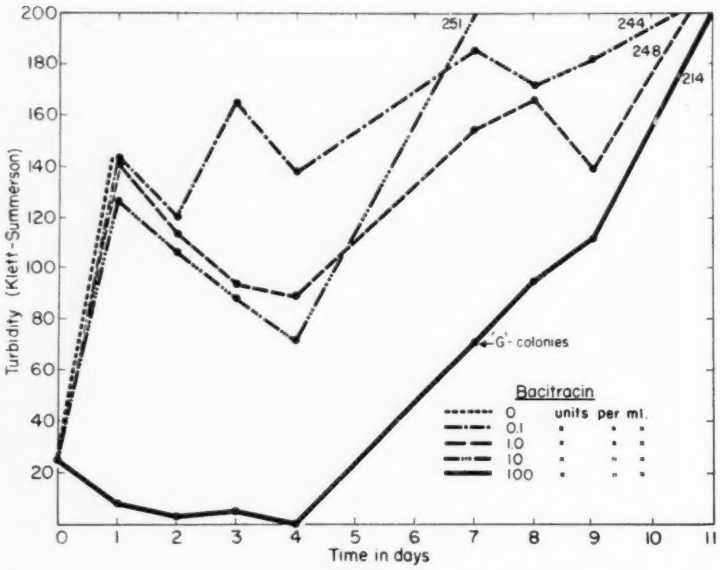

FIG. 2. INFLUENCE OF VARYING CONCENTRATIONS OF BACITRACIN UPON BROTH CULTURES OF STAPHYLOCOCCUS

Note that lysis of the organisms occurred only with the highest concentration of bacitracin—100 units per ml. G colonies were isolated only from the tube displaying lysis.

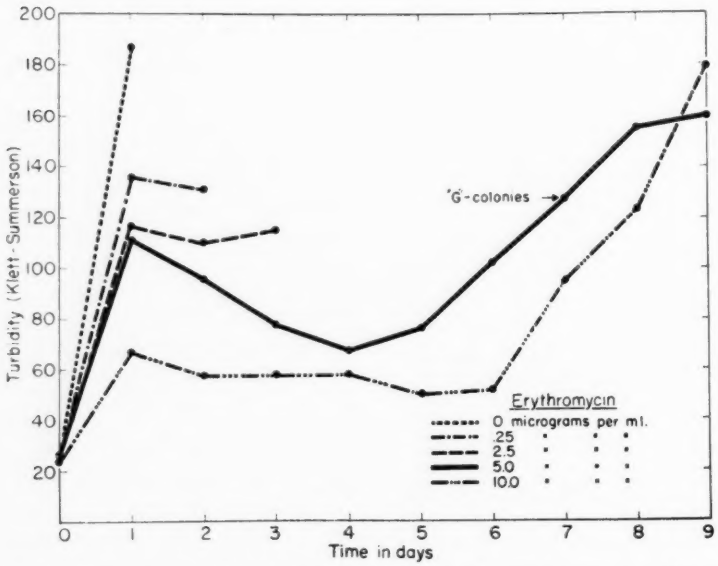

FIG. 3. INFLUENCE OF VARYING CONCENTRATIONS OF ERYTHROMYCIN UPON BROTH CULTURES OF STAPHYLOCOCCUS

Lysis of the culture was followed by the appearance of G colonies in the tube having a concentration of 5 mcg. per ml.

tures was characteristic, though different for each antibiotic. With penicillin (Figure 1) the curves of turbidity showed lysis occurring in all tubes. In agreement with Kirby (1), this took place more completely in the presence of the lower concentrations of antibiotic. After an initial clearing of the broth because of the lysis of the staphylococci, turbidity began to increase on the eighth day in the broth containing 0.1 unit per ml. The subculture from this tube showed hundreds of G colonies.

In the presence of bacitracin (Figure 2) rapid growth of the parent culture occurred in the lower concentrations, whereas lysis took place in the tube containing 100 units per ml. followed by the appearance of G cultures in this tube on the seventh day.

In the case of erythromycin (Figure 3) the bacterial turbidity increased for one day with partial lysis occurring over a period of five to seven days, when small colony variants began to appear.

With carbomycin (Figure 4) G colonies were obtained on the ninth day in the tube containing 10 micrograms per ml.

Since it had been demonstrated that G colonies could be selected out with different antibiotics, comparative studies were made with organisms from large colonies, G colonies and reverted large colonies. These investigations included a study of the general characteristics, susceptibility to bacteriophage, sensitivity to the antibiotics, production of penicillinase and virulence for small animals. Because reversion of the G colonies to large colonies occurred readily, carefully controlled measures had to be employed to detect whether G colonies or reverted large colonies were being used in the initial inoculum. This was done by inoculating the surface of agar plates with a portion of the broth culture used as the initial inoculum. Thus, after an incubation period of 24 hours, the composition of the culture used at the beginning of an experiment could be ascertained. Similarly, since reversions could occur during the period of observation both *in vitro* and *in vivo*, subcultures of the contents of tubes and material from animals were made at the conclusion of the experiment. Only in this way could significant observations be interpreted.

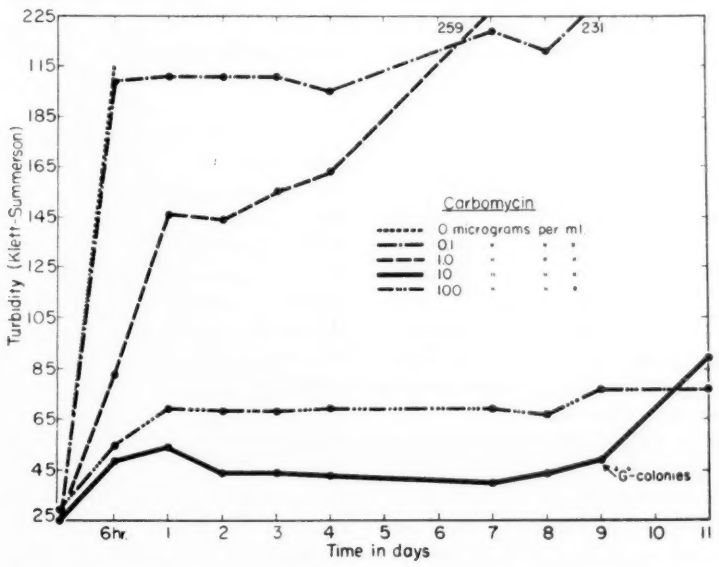

FIG. 4. INFLUENCE OF VARYING CONCENTRATIONS OF CARBOMYCIN UPON BROTH CULTURES OF STAPHYLOCOCCUS

Lysis of the organisms in a concentration of 10 mcg. per ml. was followed by the appearance of G colonies.

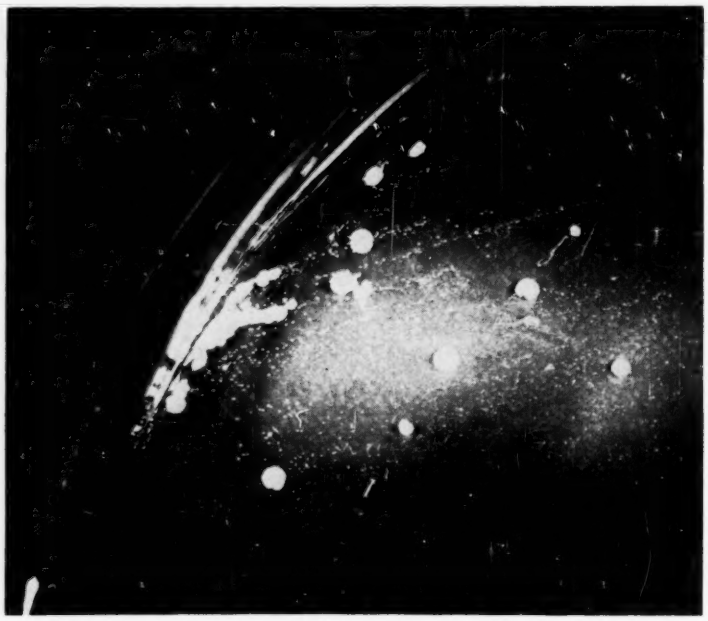

FIG. 5. SHOWING COMPARATIVE SIZE OF G COLONIES AND NORMAL-SIZED LARGE COLONIES OF STAPHYLOCOCCI

Small colonies in the presence of larger reverted colonies of normal size are demonstrated in Figure 5. The G colonies were translucent and nonpigmented, having a diameter of less than 0.1 mm., and were seen with difficulty except by reflected light or with magnification. They could be easily overlooked. When cultivated in the absence of antibiotics, they reverted to colonies of normal appearance. The reversion proceeded at slow or rapid rates; some cultures reverted almost completely within 24 hours, while others remained stable for periods of a month or more. The rate of reversion was characteristic of the strain.

Contrary to the report of Youmans and Delves (19), G colonies could readily be produced from reverted cultures, and with much more success than they could be obtained from normal parent cultures. The method used for procuring G cultures (G_2) from reverted cultures (RV) was the same as that employed for determining sensitivity of bacterial cultures to antibiotics with the tube-dilution method (50). This consisted of adding 0.5 ml. of a 1–100 dilution of an 18-hour broth culture of the reverted strain to test tubes containing 0.5 ml. of twofold serial dilutions of the antibiotic. After incubation of 24 hours, subcultures from the tubes containing the lowest concentration of antibiotic in which no bacterial growth was visible showed the presence of G colonies (G_2) in most instances.

The stained cells of G cultures of staphylococci were usually indistinguishable from those of the parent cultures in regard to uniformity in size, shape, and tinctorial properties. Some G strains showed pleomorphism with large swollen cells. The G cultures did not show any evidence of growth in Gladstone's synthetic medium, whereas this medium did support growth of the parent cultures (51). The G colonies were nonhemolytic. Although coagulase activity could not be demonstrated by the tube method in the usual three hours, some strains showed slight coagulase activity at 18 hours. It is possible that the coagulase activity at the end of this longer period was due to reverted cells. Penicillinase could not be demonstrated. G cultures which were adapted to grow in concentrations of penicillin up to 2000 units per ml. did not manifest penicillinase activity. With reversion of the adapted penicillin-resistant G strains to the large colony forms there was a

TABLE I

Penicillin-sensitivity of reverted cultures obtained from adapted penicillin-resistant G cultures

Strain No.	Units of penicillin per ml. inhibiting growth	
	G cultures	Reverted cultures
28	125	<3.9
28	500	0.5
32	125	1.9
36	15.6	1.9
1A9	15.6	0.125

loss of the acquired resistance (Table I). The G cultures were susceptible to the bactericidal action of human defibrinated whole blood, whereas the parent and reverted strains were resistant to this action. This was observed using a method described by Spink and Vivino (52). The cultures were incubated in blood for 24 hours at 37° C. and tested for viability. The results are shown in Table II. With reversion there was a return

TABLE II

Bactericidal action of human defibrinated whole blood on parent, G, and reverted cultures

Strain* No.	Bactericidal test† dilution of organisms				
	10^2	10^3	10^4	10^5	10^6
Parent 79	+	+	+	+	0
G 79	0	0	0	0	0
RV 79	+	+	+	+	0

* All cultures were of equal turbidity.
† + = growth.

to the original properties of the parent strain with respect to hemolysin, coagulase, pigmentation, and characteristics of growth.

Bacteriophage typing was carried out with the colonial variants to eliminate the possibility that the G or reverted cultures were contaminants, and also, to compare their susceptibility to bacteriophage action.[4] Table III shows the results of typing the parent (P), small colony variant (G) and reverted strains (RV) of strain 79, which had been treated with penicillin, and strain 12, which had been treated with erythromycin. Of 11 sets of cultures studied (a total of 33 strains), three sets (9 strains) were nontypeable. Eight sets consisting of 24 strains were typeable. Most of the G cultures showed no lysis with the diluted bacteriophages which were effective for the parent and reverted strains, but required undiluted bacteriophage for the demonstration of lysis. It is significant that sensitivity to at least one bacteriophage was retained by the G form, and that reversion was accompanied by a return to a lytic pattern comparable or identical with that of the parent strain. Thus, in the process of comparing the variants, there was no evidence of contamination of the cultures.

In an attempt to obtain more information concerning the selective action of penicillin for G colonies, a comparison of the penicillin-sensitivity of the related variants was made using the tube-dilution technique. Fifteen strains of G cultures were isolated from one parent and compared to their 15 reverted strains and duplicate cultures of the parent. Results showed growth of the parent strain to be inhibited by 0.06 units per ml.; the G cultures were inhibited in a range from 0.1 to 0.5 units per ml. and the reverted cultures from 0.06 to 0.12. This indicates that the G cultures were two to eightfold more resistant than the parent strain, and on reversion there was a return to a sensitivity similar to that of the parent.

[4] Bacteriophage studies were performed with the cooperation of Dr. John Blair, Hospital for Joint Diseases, New York.

TABLE III

Comparative results of typing variants of two strains of staphylococcus with bacteriophages *

Source of culture	Bacteriophages producing lysis							
Parent—A 79	7	42C	42D			53	55	52A
G colonies—G 79			42D					
Reverted colonies—RV 79	7		42D	47B	47C	53	55	52A
Parent—12 E	42B	47C						
G colonies—G 12E	42B							
Reverted colonies—RV 12E	42B	47C						

* The parent culture of strain 79 had been treated with penicillin, and that of strain 12 with erythromycin.

which confirms the observations of others (20, 38). On the other hand, Hale (21) reported that the G colony was four times more sensitive to penicillin than the parent strain. Sensitivity of related variants in which the G culture had been selected with penicillin showed no significant differences in sensitivity to chlortetracycline. In comparing related variants in similar studies with erythromycin, carbomycin, and bacitracin, there was observed a two to threefold increase in resistance of the G cultures over the parent strains; however, on reversion a decrease in resistance could not be adequately demonstrated since, as indicated previously, the reverted strains produced many G colonies when placed in the antibiotics.

Comparative virulence studies with related variants were carried out by injecting the strains intradermally into rabbits and noting the skin reactions, and intravenously into mice and observing the lethal effect (53). For these studies all cultures were incubated 24 hours in tryptose phosphate broth. To standardize the inocula for injection, the parent and reverted cultures were diluted with sterile broth to a degree of turbidity equal to the turbidity of the G culture. The number of bacteria could not be determined in the G cultures since subsurface colonies could not be readily seen with the plate method. The inoculum of the diluted parent and reverted cultures, however, contained from 200,000,000 to 1,200,000,000 cells per ml. All intradermal and intravenous injections consisted of 0.25 ml.

In the rabbit the sites of intradermal injections of the G cultures were just discernible after 48 hours. But at the sites of injections of the parent and reverted strains, there were edema, erythema, and pustule formation, and after four to six days areas of necrosis appeared. The reverted strains produced necrotic reactions in the skin that were of similar or greater severity than the reaction produced by the related parent strains.

The lethal results following the intravenous inoculation of mice are charted in Figure 6. Twenty white mice of the same age, weight, and genetic strain were injected with each culture of the related variants. At the end of 120 hours there was 100 per cent survival of mice receiving the G culture. These mice continued in apparent good health for two weeks, at which time they were sacrificed and G cultures were isolated from their kidneys. At the end of 24 hours the fatality rates of the "reverted" and "parent" groups were 90 per cent and 25 per cent, respectively. All mice

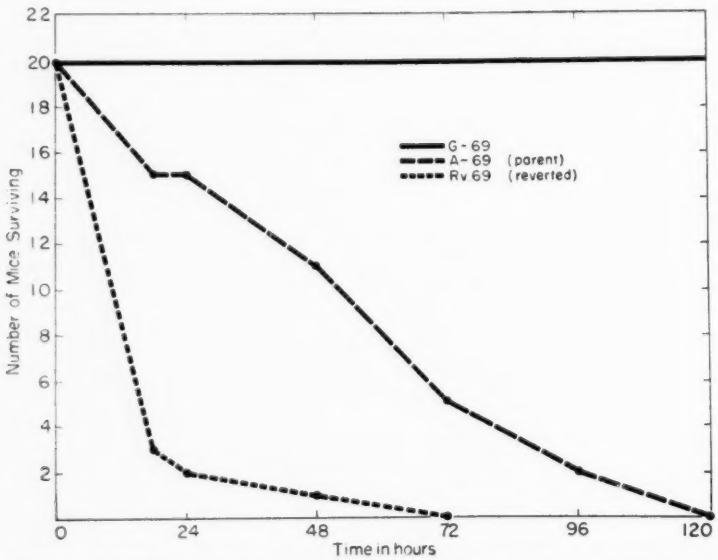

FIG. 6. SURVIVAL CURVES OF MICE FOLLOWING THE INTRAVENOUS INJECTION OF PARENT, G AND REVERTED CULTURES OF A STRAIN OF STAPHYLOCOCCUS

receiving the reverted strain were dead in 72 hours and all receiving the parent strain were dead in 120 hours. Cultures of the spleens, livers, and kidneys of mice which had been injected with the parent and reverted strains revealed staphylococci in great numbers. The outstanding features of these studies were first, the increased virulence which occurred with reversion of the avirulent small-colony variants, and second, the continued existence of the G cultures in the kidneys of apparently normal mice.

The presence of G colonies of staphylococci in human material was investigated by examining with magnification the original cultures of approximately 500 specimens of blood, exudate, urine, and other body fluids. Ten cultures of G colonies were isolated from eight patients, as shown in Table IV. Seven of the patients were being

TABLE IV

Isolation of G colonies of staphylococcus from human patients

No. of culture	Source of G colonies	Treatment received by patients
1	Fluid from chest cavity	Penicillin
2	Urine	Gantrisin
3	Throat	Penicillin Streptomycin
4	Throat	Penicillin Streptomycin
5	Urine	Chlortetracycline
6	Urine	Gantrisin Chlortetracycline
7	Blood	Penicillin
8	Urine	None

treated with an antibiotic or sulfonamide at the time the isolates were made. When the first eight cultures were examined, there was no knowledge of the identity of the specimens or the history of the patient. It is interesting that cultures 3 and 4 were from the same patient. Gram stains of G cultures 1 to 8 inclusive revealed Gram-positive cocci. These cultures gradually reverted to colonies of normal size. The characteristics of cultures 9 and 10 differed from the others and were obtained under the following circumstances: Patient C. C. was a 77 year old male with cystitis caused by *Staphylococcus aureus,* which appeared to be present in pure culture. An attempt was then made to study the production of G colonies *in vivo* in this patient. He was treated with erythromycin, 250 mg. every six hours. Urine was collected daily for culture, and the plates were examined carefully for G colonies with the aid of magnification. There was a gradual elimination of normal staphylococcal colonies over a period of four days. On the fifth day there were two normal colonies and many microcolonies which could be seen only with magnification. These small colonies were transferred to broth and to agar. Stained smears of these colonies revealed Gram-positive pleomorphic rods with cocco-bacillary and coccoid forms similar to those described by Voureka (46), which she had isolated from urine during therapy with chloramphenicol. These colonies remained stable through many subcultures and did not revert to large colonies.

DISCUSSION

It has long been known that bacteria produce small colony variants and that these forms can be recovered more readily when the cultures are subjected to unfavorable environments. In the present studies, G cultures have appeared under *in vitro* experimental conditions, and in patients following exposure of bacteria to antibacterial agents. Under *in vitro* conditions, there exists an optimum concentration of antibiotic for recovering G colonies. The G forms are two to eight times more resistant than are the parent cells. Therefore, the slow growing G variants can be isolated most readily with concentrations of antibiotic that eliminate or inhibit growth of the normal forms, and permit the G forms to reproduce. This optimum concentration falls within a narrow range. If the concentration is reduced, the normal forms reproduce rapidly and overgrow the less active G forms. If the concentration of antibiotic is too great, there is inhibition or elimination of the normal parent cells, as well as the G forms.

The G cultures are devoid of hemolysin and coagulase activity; there is an increase in nutritional requirements; and biochemical activity is diminished. The G variants have a loss of virulence and remain viable in animal tissues without producing signs of infection. Reversion to cultures of large colonies *in vivo* has not been proved. The small forms remain stable in the presence of antibiotics in concentrations that do not destroy

them. When cultivated in the absence of antibiotics, G colonies revert to large colonies, and there is a restoration of the reproduction of hemolysin and coagulase. The reverted form is as virulent as the original parent strain, and in some cases is more virulent. The reverted strain is similar but not identical to the parent, because the reverted strain may display increased virulence, and G colonies are obtained from reverted cultures more readily than they can be selected from the parent. Bacteriophage typing of related strains proved their genetic relationship and eliminated the possibility that these cultures were contaminants.

Small colony variants have been isolated from human sources, in which the patients with one exception gave a history of therapy with antibiotics or sulfonamides. It is not unlikely that optimum concentrations of antibiotics for the survival of G colonies may occur in human tissues during therapy of staphylococcal infections, and under these conditions avirulent small-colony variants may be selected out, and remain undetected. Following cessation of therapy, reversion to a virulent form may occur, and relapse of the infection may ensue. Careful bacteriological studies should include a search for G colonies subsequent to apparently successful treatment in patients with staphylococcal disease. Further investigations are now under way to determine how long the G variants will remain in tissues and under what conditions reversion to the large virulent colonies will occur.

CONCLUSIONS

1. Small colony variants (G variants) of staphylococci were selected out with the aid of antibiotics.

2. The characteristics of the G variants were studied and they are as follows: Compared to the parent strain, they have less hemolytic activity and produce less coagulase. The nutritional requirements are more demanding. The G cultures are less virulent, and remain viable in tissues of apparently normal animals. The G cultures were two to eightfold more resistant to the antibiotics than the parent cultures.

3. Reversion of G cultures to large colonies took place when cultivated in the absence of antibiotics. The characteristics of the reverted forms were the same as the parent cells, except the virulence of the reverted forms was increased in some instances.

4. Bacteriophage typing of the parent cells, and related G forms and reverted large colonies, showed a genetic relationship, which ruled out the possibilities of contamination.

5. Cultures of G colonies were obtained from human sources following treatment with antibacterial drugs.

REFERENCES

1. Kirby, W. M. M., Bacteriostatic and lytic actions of penicillin on sensitive and resistant staphylococci. J. Clin. Invest., 1945, **24**, 165.
2. Hadley, P., Delves, E., and Klimek, J., The filterable forms of bacteria. I. A filterable stage in the life history of the Shiga dysentery bacillus. J. Infect. Dis., 1931, **48**, 1.
3. Jacobsen, K. A., Mitteilungen über einen variablen Typhusstamm (Bacterium typhi mutabile), sowie über eine eigentumliche hemmende Wirkung des gewöhnlichen Agar, verursacht durch Autoklavierung. Centralb. f. Bakt. 1910, Abt. 1, **56**, 208.
4. Eisenberg, P., Untersuchungen über die Variabilität der Bakterien. IV. Ueber den Variationskreis des B. prodigiosum und B. violaceum. Centralb. f. Bakt., 1914, Abt. 1, **73**, 449 and 466.
5. Baerthlein, K., Ueber bakterielle Variabilität, insbesondere sogenannte Bakterienmutationen. Centralb. f. Bakt., 1918, 1, **81**, 369.
6. Fürth, J., Variationsversuche mit Paratyphus β. (Weil). Ztschr. f. Immunitätsforsch u. exper. Therap., 1922, **35**, 155.
7. Fürth, J., Rezeptorenanalyse und Variationsversuche mit B. Paratyphus Aertryck. Ztschr. f. Immunitätsforsch u. exper. Therap., 1922, **35**, 162.
8. Friedberger, E., Ueber Bacterienvirus (kryptantigenes Virus) nach Versuchen mit Typhusvirus. Ztschr. f. Immunitätsforsch u. exper. Therap., 1927, **53**, 339.
9. Hoffstadt, R. E., and Youmans, G. P., *Staphylococcus aureus:* Dissociation and its relation to infection and to immunity. J. Infect. Dis., 1932, **51**, 216.
10. Hoffstadt, R. E., and Youmans, G. P., The genetic significance of the dissociants of *Staphylococcus aureus*. J. Bact., 1934, **27**, 551.
11. Hoffstadt, R. E., Youmans, G. P., and Clark, W., Antigenic structure of *Staphylococcus aureus* and its variants. J. Bact., 1934, **27**, 97.
12. Swingle, E. L., Studies on small colony variants of *Staphylococcus aureus*. Proc. Soc. Exper. Biol. & Med., 1934, **31**, 981.

13. Youmans, G. P., Production of small-colony variants of *Staphylococcus aureus*. Proc. Soc. Exper. Biol. & Med., 1937, **36**, 94.
14. Rettger, L. F., and Gillespie, H. B., Bacterial variation, with special reference to pleomorphism and filterability. J. Bact., 1933, **26**, 289.
15. Hadley, P., and Carapetian, H., A study of the infective qualities possessed by G phase cultures of *Bacillus typhosus*. J. Bact., 1933, **25**, 94.
16. Raney, M. E., and Kopeloff, N., Dissociation and filtration studies with *L. acidophilus*. J. Bact., 1934, **27**, 45.
17. Weinberg, E. D., Vitamin requirements of dwarf colony variants of bacteria. J. Infect. Dis., 1950, **87**, 299.
18. Duff, D. C. B., Dissociation in *Bacillus salmonicida*, with special reference to the appearance of a G form of culture. J. Bact., 1937, **34**, 49.
19. Youmans, G. P., and Delves, E., The effect of inorganic salts on the production of small colony variants by *Staphylococcus aureus*. J. Bact., 1942, **44**, 127.
20. Schnitzer, R. J., Camagni, L. J., and Buck, M., Resistance of small colony variants (G-forms) of a staphylococcus toward the bacteriostatic activity of penicillin. Proc. Soc. Exper. Biol. & Med., 1943, **53**, 75.
21. Hale, J. H., Studies on staphylococcus mutation: Characteristics of the "G" (gonidial) variant and factors concerned in its production. Brit. J. Exper. Path., 1947, **28**, 202.
22. Colwell, C. A., Small colony variants of *Escherichia coli*. J. Bact., 1946, **52**, 417.
23. Hoffstadt, R. E., and Almaden, P., Dissociation of *Staphylococcus aureus*. III. Relation of bacteriophage to the dissociation of *Staphylococcus aureus*. J. Infect. Dis., 1934, **54**, 253.
24. Haddow, A., Small-colony variation in B. paratyphosus B (Tidy) and other bacteria, with special reference to the G type of Hadley. J. Infect. Dis., 1938, **63**, 129.
25. Novak, M. V., and Henrici, A. T., Pleomorphic organism showing relationships between staphylococci and actinomycetes. J. Infect. Dis., 1933, **52**, 253.
26. Swingle, E. L., Studies on small colony variants of *Staphylococcus aureus*. J. Bact., 1935, **29**, 467.
27. Chinn, B. D., Characteristics of small colony variants of *Shigella paradysenteriae Sonne* and *Staphylococcus aureus*. Proc. Soc. Exper. Biol. & Med., 1936, **34**, 237.
28. Colien, F. E., A study of microbic variation in a yellow pigment-producing coccus. J. Bact., 1935, **30**, 301.
29. Dienst, R. B., A study of some of the factors promoting dissociation of *Bacterium dysenteriae Sonne*. J. Bact., 1933, **26**, 489.
30. Koser, S. A., and Dienst, R. B., Small colony variants or G forms of *Eberthella dysenteriae Sonne*. J. Infect. Dis., 1934, **54**, 131.
31. Chinn, B. D., Characteristics of small colony variants with special reference to *Shigella paradysenteriae Sonne*. J. Infect. Dis., 1936, **59**, 137.
32. Roe, A. F., Dissociation of *Cl. welchii*. J. Bact., 1934, **27**, 46.
33. Kopeloff, N., Dissociation and filtration of *Lactobacillus acidophilus*. J. Infect. Dis., 1934, **55**, 368.
34. Raven, C., Dissociation of the gonococcus. J. Infect. Dis., 1934, **55**, 328.
35. Raven, C., Dissociation of the gonococcus. Proc. Soc. Exper. Biol. & Med., 1934, **31**, 899.
36. Fabian, F. W., and McCullough, N. B., Dissociation in yeasts. J. Bact., 1934, **27**, 43.
37. Huddleson, I. F., and Baltzer, B., The characteristics and dissociation pattern of type G (micro-colony type) of *Brucella abortus in* Studies in Brucellosis, III. A series of five papers. East Lansing, Michigan State College, Agr. Exp. Sta., Department of Bacteriology and Public Health, 1952, p. 64.
38. Youmans, G. P., Williston, E. H., and Simon, M., Production of small colony variants of *Staphlococcus aureus* by the action of penicillin. Proc. Soc. Exper. Biol. & Med., 1945, **58**, 56.
39. Eriksen, K., Studies on induced resistance to penicillin in staphylococci. Acta path. et microbiol. Scandinav., 1946, **23**, 284.
40. Barbour, R. G. H., Small colony variants ("G" forms) produced by *Staph. pyogenes* during the development of resistance to streptomycin. Australian J. Exper. Biol. & M. Sc., 1950, **28**, 411.
41. Edwards, P. R., Studies on *Shigella equirulis*. III. The occurrence of dwarf colony variants. Kentucky Agr. Exp. Sta. Bull., 1931, No. **320**, 320.
42. Bliss, E. A., and Long, P. H., Studies on minute hemolytic streptococci. I. The cultural characteristics of minute hemolytic streptococci. J. Bact., 1934, **27**, 105.
43. Morton, H. E., and Shoemaker, J., The identification of *Neisseria gonorrhoeae* by means of bacterial variation and the detection of small colony forms in clinical material. J. Bact., 1945, **50**, 585.
44. Morris, J. F., Sellers, T. F., and Brown, A. W., The primary isolation of small colony strains of *Eberthella typhosa* from blood, feces, urine, and sputum. J. Infect. Dis., 1941, **68**, 117.
45. Hall, W. H., and Spink, W. W., *In vitro* sensitivity of *Brucella* to streptomycin: Development of resistance during streptomycin treatment. Proc. Soc. Exper. Biol. & Med., 1947, **64**, 403.
46. Voureka, A., Bacterial variants in patients treated with chloramphenicol. Lancet, 1951, **i**, 27.
47. Hale, J. H., Studies on staphylococcus mutation: A naturally occurring "G" gonidial variant and its

carbon dioxide requirements. Brit. J. Exper. Path., 1951, **32**, 307.

48. Sherris, J. C., Two small colony variants of *Staph. aureus* isolated in pure culture from closed infected lesions and their carbon dioxide requirements. J. Clin. Path., 1952, **5**, 354.
49. Lindegren, C. C., Genetical studies of bacteria. III. Origin of G-type colonies by transgenation. Zentralbl. f. Bakt., II, 1935, **93**, 389.
50. Waisbren, B. A., Carr, C., and Dunnette, J., The tube dilution method of determining bacterial sensitivity to antibiotics. Am. J. Clin. Path., 1951, **21**, 884.
51. Gladstone, G. P., The nutrition of *Staphylococcus aureus:* nitrogen requirements. Brit. J. Exper. Path., 1937, **18**, 322.
52. Spink, W. W., and Vivino, J. J., The coagulase test for staphylococci and its correlation with the resistance of the organisms to the bactericidal action of human blood. J. Clin. Invest., 1942, **21**, 353.
53. Gorrill, R. H., Experimental staphylococcal infections in mice. Brit. J. Exper. Path., 1951, **32**, 151.

PHOSPHORUS EXCRETION IN RENAL FAILURE [1]

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The kidney provides a sensitive mechanism which aids in the maintenance of phosphorus equilibrium despite varying intakes and alterations in metabolic demand. Under normal conditions a large proportion of the phosphorus which is filtered at the glomerulus is reabsorbed by the tubules which thereby effect regulation of the urinary phosphorus (1). Few studies have been performed on patients or animals to determine how phosphorus homeostasis is maintained as renal function is progressively decreased (2, 3). It is well known that the concentration of serum inorganic phosphorus increases and of calcium decreases as renal failure becomes more pronounced (4–6), and that anatomical changes occur in the parathyroid glands which suggest hyperfunction of these organs (7). Since the parenteral administration of parathyroid hormone produces an increased phosphaturia, a decrease in the serum phosphorus, and an increase in the serum calcium, it is possible that the parathyroid hyperplasia represents a physiologic attempt to reverse these specific effects of renal failure (8). It seemed appropriate, therefore, to investigate the relationship of phosphate clearance to the general level of renal function and to test the response to exogenous parathyroid hormone at all gradations of renal impairment to determine whether a further response was possible or whether maximal effectiveness had been achieved by endogenous hormone activity.

MATERIALS AND METHODS

A series of twenty males between the ages of 25 and 60 years was selected. Their glomerular filtration rates as measured by inulin clearance varied from 142 to 1.5 ml. per minute, and covered the entire range of renal function from normal to advanced uremia. Those with reduced renal function had various diseases, chiefly chronic glomerulonephritis and chronic pyelonephritis. The patients with normal kidneys had been maintained on the regular hospital diet which provided 90 to 100 grams of protein and about 1.5 grams of phosphorus per day. Most of the patients with reduced renal function had ingested a diet in which the protein was restricted to about 40 grams and the phosphorus intake to approximately 0.75 grams per day.

Clearances were performed during the postabsorptive state and the glomerular filtration rate was measured by the use of inulin in all but four experiments in which the endogenous creatinine clearance was substituted. Three control periods of approximately 20 minutes each were obtained 30 minutes after the injection of the priming dose of inulin. Blood was drawn for the determination of serum inulin and phosphorus at the midpoint of each clearance period. During the fourth 20-minute period 500 units of parathyroid hormone [2] (Lilly) was given by slow intravenous injection. Three additional 20-minute clearance periods were obtained following the administration of the hormone. A satisfactory rate of urine flow was maintained by the administration of 500 ml. of fluid in the inulin-sustaining solution and by the ingestion of about 200 ml. of water every hour. Completeness of urine collection was assured by two consecutive bladder irrigations with 20 ml. of distilled water followed by 20 ml. of air. The concentration of inulin in the plasma and urine was determined by the resorcinol method of Schreiner (9), creatinine by the method of Bonsnes and Taussky (10), and phosphorus by the method of Fiske and SubbaRow (11).

Glomerular filtration of phosphorus (GFP) was assumed to be the product of the serum inorganic phosporus concentration and the glomerular filtration rate, and has been expressed in milligrams of phosphorus filtered per minute. The filtered phosphorus which did not appear in the urine presumably was reabsorbed by the tubules (TRP). The TRP is thus the difference between the GFP and the urinary phosphorus (UP).

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[2] The evening before the clearances were performed, 0.1 ml. of a 1:1000 dilution of parathyroid hormone was injected intradermally as a test for possible sensitivity. The few patients who showed positive skin reactions were excluded.

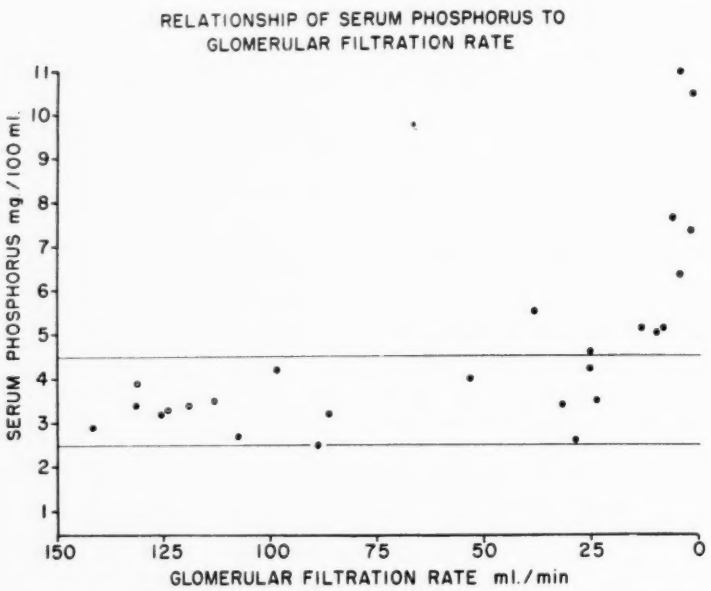

FIG. 1. THE RELATIONSHIP OF THE SERUM PHOSPHORUS TO THE GLOMERULAR FILTRATION RATE

The open circles are derived from the data of Kleeman and Cooke (2).

TABLE I

Renal excretion of phosphorus at varying levels of renal function **

Patient	Age	Serum P *mg./ml.*	Urine vol. *ml./min.*	Urine P *mg./min.*	GFR *ml./min.*	GFP *mg./min.*	TRP *mg./min.*
R. N. L.	54	0.030	1.60	0.19	141	4.20	4.01
E. A. B.	43	0.033	1.07	0.31	129	4.20	3.89
E. L. S.	26	0.032	4.88	0.33	123†	3.86	3.54
G. T. H.	41	0.027	5.26	0.17	97.4	2.61	2.44
R. S.	51	0.043	2.87	0.47	86.7	3.72	3.25
T. K.	55	0.032	1.50	0.80	83.5	2.69	1.89
W. A. B.	66	0.024	3.29	0.41	89.0	2.15	1.74
J. G. G.	40	0.039	6.42	0.80	53.0	2.06	1.25
K. R.	33	0.058	2.36	0.64	38.1	2.19	1.55
S. T.	34	0.033	2.94	0.45	32.2	1.06	0.61
G. M. D.	26	0.025	3.01	0.45	27.6	0.70	0.25
I. R. D.	37	0.035	1.21	0.41	23.8	0.82	0.42
R. S.	41	0.043	0.85	0.48	23.6	1.02	0.54
E. S.	31	0.045	1.45	0.36	25.7†	1.16	0.80
G. O.	54	0.053	2.08	0.61	14.2	0.75	0.14
J. R. T.	56	0.048	2.47	0.28	9.0	0.43	0.15
C. E. D.	65	0.052	0.90	0.36	8.5	0.45	0.09
C. A.	53	0.077	2.40	0.52	6.3†	0.49	−0.03‡
R. C.	23	0.107	1.95	0.45	4.7†	0.50	0.05
D. E. B.	54	0.104	0.30	0.15	1.5	0.16	0.01

* UP—Urine phosphorus.
GFR—Glomerular filtration rate.
GFP—Glomerular filtrate phosphorus.
TRP—Tubularly reabsorbed phosphorus.
† Creatinine clearance.
‡ UP exceeded GFP by amount indicated.

RESULTS

The data are summarized in the tables and illustrated in two figures. With but one exception, the serum phosphorus remained within the normal range of 2.5 to 4.5 mg. per cent, until the filtration rate was reduced to 25 ml. per minute or below. At filtration rates of less than 15 ml. per minute, none of the serum phosphorus values were normal and they increased markedly as filtration was further reduced. These data have been plotted in Figure 1 which includes a few values (open circles) obtained by Kleeman and Cooke (*2*).

It is to be noted that there was some reabsorption of phosphorus except possibly in the final stages of renal insufficiency (Tables I and II). There were actually three observations in two uremic patients in which more phosphorus was excreted than was filtered, a circumstance which could occur only if there was some excretion of phosphorus by the tubules. We are inclined to minimize this possibility because in two of the three instances in which the excretion of phosphate exceeded the calculated amount filtered, the filtration rates are based on creatinine clearances. That PTH increased the excretion of urinary phosphorus was apparent in every subject down to and including one with a filtration rate of 10.6 ml. per minute. Below this point UP so closely approximated GFP that the effect of the hormone was no longer evident. The decreasing response to PTH as renal function declines is visualized in Figure 2, where the ratios $UP_2 : UP_1$ (after and before PTH administration) are plotted against the GFR. Although there was some response to parathyroid hormone at all levels of function, it should be stressed that this became quite small even prior to gross impairment of glomerular filtration. Substantial increases in urinary phosphorus were observed in four patients whose filtration rates were above 100 ml. per minute, but there was only one instance in which as much as a twofold increase in UP occurred at a lower filtration rate. The absolute amount of phosphorus excreted following PTH was always small, and in only two subjects did it exceed 1.0 mg. per minute. Therefore, the high ratios of $UP_2 : UP_1$ were dependent upon a very low rate of phosphorus excretion during the control periods.

The factors participating in the production of phosphaturia were an increase in GFR, a decrease in TRP and a combination of these two. In patients with markedly impaired renal function both responses were greatly reduced or absent (Table

TABLE II

Renal excretion of phosphorus after parathyroid hormone administration *

Patient	Serum P *mg./ml.*	Urine vol. *ml./min.*	Urine P *mg./min.*	GFR *ml./min.*	GFP *mg./min.*	TRP *mg./min.*
R. N. L.	0.029	8.40	0.95	142	4.08	3.13
E. A. B.	0.035	1.15	0.91	136	4.74	3.83
E. L. S.	0.031	7.60	0.83	128†	3.98	3.14
G. T. H.	0.027	4.49	0.69	118	3.18	2.49
R. S.	0.040	4.51	0.86	110.5	4.44	3.58
T. K.	0.032	3.85	1.03	89.8	2.87	1.84
W. A. B.	0.025	6.83	0.68	89.1	2.23	1.55
J. G. G.	0.040	4.31	1.10	54.4	2.18	1.08
K. R.	0.052	5.50	0.66	38.6	2.01	1.35
S. T.	0.034	3.87	0.57	31.7	1.08	0.51
G. M. D.	0.027	4.28	0.62	30.9	0.82	0.21
I. R. D.	0.035	2.30	0.52	24.6	0.85	0.33
R. S.	0.040	1.52	0.64	27.2	1.08	0.44
E. S.	0.046	2.10	0.71	25.6†	1.17	0.46
G. O.	0.049	1.93	0.51	13.1	0.65	0.14
J. R. T.	0.051	4.19	0.35	10.6	0.55	0.19
C. E. D.	0.050	0.88	0.31	7.9	0.40	0.08
C. A.	0.074	1.93	0.51	6.2†	0.44	−0.07‡
R. C.	0.110	1.48	0.36	4.0†	0.44	0.07
D. E. B.	0.103	0.45	0.17	1.4	0.15	−0.01‡

* See Table I for explanation of abbreviations.
† Creatinine clearance.
‡ UP exceeded GFP by amount indicated.

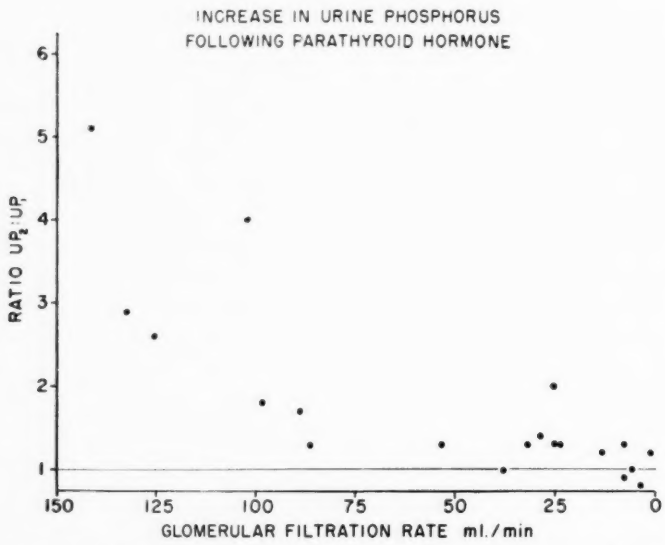

FIG. 2. THE RATIO OF THE RATE OF PHOSPHORUS EXCRETION AFTER PARATHYROID HORMONE ADMINISTRATION TO THE RATE BEFORE HORMONE ADMINISTRATION

III). Otherwise, with the exception of three cases whose clearances were above 85 ml. per minute and in whom an increase in GFR alone was responsible for the rise in urinary phosphorus, there was a measurable and apparently significant decrease in TRP.

TABLE III

Comparison of UP, GFR, and TRP before and after parathyroid hormone administration

Patient	UP_2/UP_1	$\frac{GFR_2}{GFR_1}$	$\frac{TRP_2}{TRP_1}$
R. N. L.	5.12	1.01	0.78
E. A. B.	2.89	1.05	0.99
E. L. S.	2.56	1.04	0.89
G. T. H.	3.97	1.21	1.02
R. S.	1.84	1.27	1.10
T. K.	1.28	1.08	0.97
W. A. B.	1.66	1.00	0.89
J. G. G.	1.35	1.03	0.86
K. R.	1.02	1.01	0.87
S. T.	1.27	0.99	0.84
G. M. D.	1.37	1.12	0.83
I. R. D.	1.25	1.08	0.79
R. S.	1.32	1.15	0.81
E. S.	2.00	1.00	0.58
G. O.	1.18	0.92	0.98
J. R. T.	1.25	1.18	1.30
C. E. D.	.87	0.93	0.95
C. A.	.98	0.98	
R. C.	.80	0.85	1.49
D. E. B.	1.10	0.93	

DISCUSSION

Ingestion of a normal diet containing 1500 mg. of phosphorus requires the urinary excretion of approximately 750 mg. of phosphorus daily in order to maintain metabolic balance. This is an average of 0.5 mg. per minute. At normal concentrations of serum inorganic phosphorus of 3.5 mg. per 100 ml., a minimum glomerular filtration rate of 14 ml. per minute is required to provide 0.5 mg. of filtered phosphorus. Apparently, the tubules continue to reabsorb filtered phosphorus despite inhibition by the administered PTH and the stress of renal failure. For this reason the GFP must be larger than 0.5 mg. per minute in order to maintain an average urinary phosphorus of 0.5 mg., and the serum phosphorus rises at filtration rates actually higher than 14 ml. In the more seriously ill patients, the tendency for the concentration of serum phosphorus to increase at filtration rates higher than that stipulated here, is, of course, partially offset by a reduction in the intake of phosphorus. This could, in some cases, have reduced the amount requiring excretion in the urine by as much as 50 per cent. Except for one example of an increase in serum phosphorus at a filtration rate of 38 ml., our information indi-

cates that the critical point lies between 24 and 14 ml. From the data we have accumulated it seems reasonably certain that there is no significant excretion of phosphorus by the tubules and that increases in the concentration of serum phosphorus are required when filtration falls below a minimum set by the load imposed by the dietary intake. Thus, our observations support theoretical curves presented by Gamble (12) which relate the glomerular filtration rate and the required phosphate excretion to the concentration of serum inorganic phosphorus.

The assumption that at high concentrations of serum phosphorus all of the serum inorganic phosphorus is filterable, is supported by the observation that as the GFR decreases with renal disease, GFP and UP tend to become substantially identical. Should any significant amount of the phosphorus be nonfilterable, the true GFP would be less than the amount calculated and hence one would not expect identity between UP and GFP, unless the deficit was supplied by excretion of phosphate by the tubules. The reason for excluding this latter possibility has been commented upon in the presentation of the data. Handler and Cohn (13) have recently published information based upon isotopic studies which also support the concept of complete filterability of the serum inorganic phosphorus.

Although the cause of phosphaturia following the intravenous administration of PTH appears in our experience to be about equally divided between an increased glomerular filtration of phosphorus and a decreased reabsorption by the tubules, and thus tends to substantiate certain other investigations (14, 15), it is conceivable that this dual mechanism may not represent the physiological effects of the parathyroid glands on the kidneys. The increases observed in GFR may be an artefact since it has been found by Handler and Cohn (16) that the subcutaneous injection of parathyroid extract in the dog has little effect on renal hemodynamics yet is still capable of lowering the plasma inorganic phosphate concentration and producing phosphaturia.

If it may be assumed that the response to 500 units of PTH is maximal, then the ratio of increase in phosphorus excretion ($UP_2 : UP_1$) depends upon the rate of excretion at the time of PTH injection. No method for achieving minimal excretion was attempted, so the full range of response could not be determined. By performing the studies during the morning, when minimal excretion is expected (17–19), it was hoped that maximal increases, and, therefore, the largest ratios of increase, would be observed. Only in the relatively normal individuals was there a very low pre-injection rate of excretion followed by a high rate after PTH. Thus, the normal kidney was capable of rapid changes in the rate of phosphorus excretion, a function which appeared to be lost early in renal failure. The response to PTH appears to depend upon both the integrity of this function of the kidney and a low rate of excretion during the period prior to injection. When renal function is grossly impaired, extra-renal mechanisms, notably fluctuations in the serum phosphorus level, affect regulation of phosphorus excretion.

Crawford, Osborne, Talbot, Terry, and Morrill (20) have found that in rats complete parathyroidectomy causes the TRP : GFP ratio to approach 1.0, while parathyroid hormone administration causes this ratio to approach zero. Later studies upon human subjects with presumably normal renal function showed changes in the anticipated direction after PTH administration, although not reaching the extreme values of the animal studies. The present data demonstrate changes in the same direction. However, as renal function decreases the TRP gradually decreases while the UP remains constant. In complete renal failure the TRP : GFP ratio approaches zero. It cannot be determined whether this is due to maximal physiologic activity of PTH or to a reduction in functioning tubular tissue to the point where no further reabsorption could be expected.

SUMMARY

1. The excretion of phosphorus was studied in 20 males whose renal function ranged from normal through various gradations of impairment including terminal uremia.

2. In an attempt to elucidate the mechanism of phosphorus excretion in the urine as kidney function became progressively worse, simultaneous measurements of inulin and phosphorus clearances

were performed before and after intravenous injection of 500 units of parathyroid hormone.

3. Glomerular filtration rates (GFR) varied from 142 to 1.5 ml. per minute. With but one exception, the serum phosphorus remained within the normal range of 2.5 to 4.5 mg. per cent until the GFR was reduced to about 25 ml. per minute. As GFR was further reduced the concentration of serum phosphorus increased markedly.

4. Phosphorus filtered at the glomerulus (GFP) was calculated as the product of the concentration of inorganic phosphorus in serum and the volume of glomerular filtrate. Phosphorus reabsorbed by the tubules (TRP) was assumed to be the difference between the filtered phosphorus and the urinary phosphorus (UP).

5. Evidence for TRP was found, except in terminal uremia, when GFP closely approximated UP, suggesting a quantitative transfer of phosphorus filtered at the glomeruli to the urine.

6. The administration of PTH caused an increase in UP which was apparently due both to an increase in GFR and a decrease in TRP. The responsiveness to PTH was lost in renal disease when the GFR was reduced below 10 ml. per minute. With further renal impairment it was impossible to determine whether there was a preexisting maximum effect of endogenous PTH or whether the tubules failed to respond.

REFERENCES

1. Smith, H. W., The Kidney. Structure and Function in Health and Disease. New York, Oxford University Press, 1951.
2. Kleeman, C. R., and Cooke, R. E., The acute effects of parathyroid hormone on the metabolism of endogenous phosphate. J. Lab. & Clin. Med., 1951, **38**, 112.
3. Goadby, H. K., On the action of parathormone. III. Biochem. J., 1937, **31**, 1530.
4. Marriott, W. M., and Howland, J., Phosphate retention as a factor in the production of acidosis in nephritis. Arch. Int. Med., 1916, **18**, 708.
5. Schmitz, H. W., Rhodenburg, E. L., and Myers, V. C., The inorganic phosphorus and calcium of the blood in nephritis. Arch. Int. Med., 1926, **37**, 233.
6. Schulz, I., Phosphorus in the blood and urine: a study of the excretion and retention of phosphorus in a normal subject and in patients with renal disease. Ann. Int. Med., 1930, **3**, 667.
7. Fritz, G. E., and Brines, O. A., The cell type of secondary parathyroid hyperplasia. Am. J. Path., 1951, **27**, 265.
8. Albright, F., and Reifenstein, E. C., Jr., The Parathyroid Glands and Metabolic Bone Disease. Selected Studies. Baltimore, Williams & Wilkins Co., 1948.
9. Schreiner, G. E., Determination of inulin by means of resorcinol. Proc. Soc. Exper. Biol. & Med., 1950, **74**, 117.
10. Bonsnes, R. W., and Taussky, H. H., On the colorimetric determination of creatinine by the Jaffe reaction. J. Biol. Chem., 1945, **158**, 581.
11. Fiske, C. H., and SubbaRow, Y., The colorimetric determination of phosphorus. J. Biol. Chem., 1925, **66**, 375.
12. Gamble, J. L., Chemical anatomy, physiology and pathology of extracellular fluid; A lecture syllabus, Cambridge, Harvard University Press, 1947.
13. Handler, P., and Cohn, D. V., Use of radiophosphorus in studies of glomerular permeability of plasma inorganic phosphate. Am. J. Physiol., 1951, **164**, 646.
14. Jacobs, E., and Verbanck, M., Parathormone et réabsorption rénale du phosphore. J. d'urol., méd. et chir., 1952, **58**, 244.
15. Klein, R., and Gow, R. C., Interaction of parathyroid hormone and vitamin D on the renal excretion of phosphate. J. Clin. Endocrinol. & Metab., 1953, **13**, 271.
16. Handler, P., and Cohn, D. V., Effect of parathyroid extract on renal function. Am. J. Physiol., 1952, **169**, 188.
17. Ollayos, R. W., and Winkler, A. W., Urinary excretion and serum concentration of inorganic phosphate in man. J. Clin. Invest., 1943, **22**, 147.
18. McCorvie, J. E., Studies on the morning alkaline tide of urine in normal persons and in patients with nephritis. J. Clin. Invest., 1925–26, **2**, 35.
19. Stanbury, S. W., and Thomson, A. E., Diurnal variations in electrolyte excretion. Clin. Sc., 1951, **10**, 267.
20. Crawford, J. D., Osborne, M. M., Jr., Talbot, N. B., Terry, M. L., and Morrill, M. F., The parathyroid glands and phosphorus homeostasis. J. Clin. Invest., 1950, **29**, 1448.

THE GLUCAGON CONTENT OF CRYSTALLINE INSULIN PREPARATIONS

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Shortly after the discovery of insulin, investigators reported that intravenous administration of this hormone leads to a transient hyperglycemia. Kimball and Murlin (1) suggested that this response was caused by the presence of a second physiologically active substance which they named glucagon. Some workers (2, 3), however, suggested that this effect might be ascribed to insulin, or possibly to an artifact created during the manufacture of insulin (4). Considerable evidence accumulated during the last decade favors the view that the short initial hyperglycemia observed following administration of insulin preparations, is due to the presence of small quantities of a second active substance, probably secreted by the α-cells of the pancreatic islets. The successful isolation of the hyperglycemic principle in highly purified and crystalline form (5) furnished conclusive proof that the factor is indeed an entity different from insulin. The amino acid composition of the crystalline material differs significantly from that of insulin (6). Available evidence indicates that the two proteins also differ in regard to their physico-chemical properties. Hence, it appears unlikely that difficulties in separating glucagon from insulin are due to similar chemical or physico-chemical properties as concluded by Bürger and Brandt (7). Although most of the hyperglycemic principle is removed from insulin in the course of its purification, small amounts can be found in most purified preparations. Many of these have been described as homogeneous on the basis of physico-chemical evidence (8). However, more recent work, based on counter current distribution (9), electrophoresis (10), and electrophoresis convection (11), have suggested the presence of some heterogeneity in crystalline insulin preparations. Small amounts of glucagon might partly account for these findings. Several authors have attempted to estimate the glucagon content of various crystalline insulin preparations (10, 12–14). These estimates, ranging from 1 to 10 per cent, have been obtained by indirect or at best semi-quantitative methods and must be regarded as tentative. The uncertainty of these results prompted the present quantitative study of the glucagon content of crystalline insulin samples. For this purpose the hyperglycemic activity of a number of different insulin preparations was compared with that of reference samples consisting of glucagon-free insulin to which known amounts of crystalline glucagon had been added. The results of these experiments are described in this paper.

BIOLOGICAL ASSAY

The method for measuring the biological activity of glucagon is based on the hyperglycemic response in cats (15). This animal was selected because of its high sensitivity and comparatively constant response. Interference by the hypoglycemic action of insulin during the biological assay was prevented by incubating the samples with cysteine. This procedure destroys insulin activity but has no effect on the glucagon.

Starved cats (16 to 18 hours) weighing 2500 to 3000 grams were anesthetized by intraperitoneal injection of 70 to 80 mg. of Amytal Sodium® (Amobarbital Sodium, Lilly) per Kg. After 30 minutes the femoral vein was exposed in both legs. One vein was used for injection of the test solution (administered in a volume of 0.05 ml. per Kg.) and the other for blood sampling. A control blood sample was collected before injection of the test solution; additional samples were withdrawn at five-minute intervals during 25 minutes. Blood sugar was determined according to the method of Somogyi (16).

Administration of increasing quantities of glucagon produces a proportional elevation of the blood sugar level until a maximum response is obtained. Larger doses have no additional effect on the net elevation of the blood sugar but cause a prolongation of the hyperglycemia. The maximum response in the cat, a blood sugar increase of approximately 100 mg. per cent, is ob-

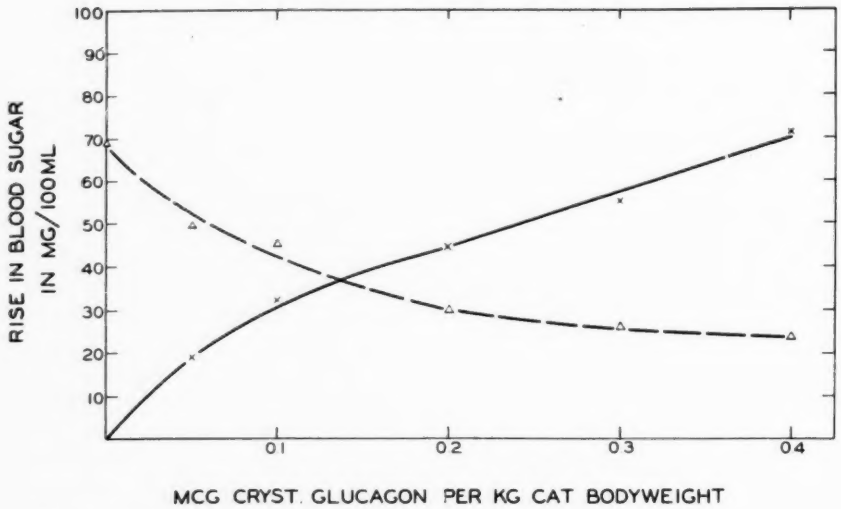

FIG. 1. EFFECT OF INCREASING DOSES OF GLUCAGON ON THE BLOOD SUGAR LEVEL AND THE STANDARD RESPONSE

——×——×—— Maximum rise in blood sugar caused by crystalline glucagon (first injection).

——△——△—— Maximum hyperglycemia caused by the standard injected 25 minutes after administration of the crystalline glucagon.

tained by intravenous injection of 1 to 2 mcg. per Kg. of crystalline glucagon.

Figure 1 shows the increase in blood sugar as a function of the glucagon dose. Experience has shown that greatest reproducibility is obtained in the range below half maximum response. For this reason the protein concentration of the test dose was adjusted so that the blood sugar rise was within the limits of 20 to 50 mg. per cent. Under these conditions maximum hyperglycemia occurs between 10 and 15 minutes following injection.

The standard deviations of column 3 in Table II show that the net blood sugar increase, caused by a given dose of glucagon, varies within relatively wide limits. A typical example of the variation in sensitivity that may be expected is given in Table I.

Information about the sensitivity of each particular animal is gained by injecting a standard preparation of known potency immediately following the first 25-minute interval. Since the hyperglycemic peak again occurs between 10 and 15 minutes following injection, the second half of the test need not be extended beyond 15 minutes. Column 2 in Table I shows that the blood sugar rise caused by the standard varies approximately in proportion to the rise obtained by the sample injected initially. This makes it possible largely to eliminate the variable due to sensitivity

TABLE I

Variation of blood sugar in cats in response to a given dose of glucagon

Cat No.	Maximum rise in blood sugar in *mg. per 100 ml.*	
	First injection 0.05 mcg. cryst. glucagon per Kg.	Second injection* 0.05 ml. of standard per Kg.
1	9.1	36.2
2	22.6	66.7
3	11.3	49.8
4	20.3	55.3
5	22.6	75.7
6	28.3	44.6
7	24.3	50.3
8	18.6	49.7
9	11.8	45.8
10	10.2	23.2
11	11.8	43.5
12	4.5	17.3
13	30.0	79.6

* The standard is injected immediately after withdrawal of the 25-minute blood sample. It is equivalent to 0.2 mcg. crystalline glucagon per Kg.

TABLE II

Response ratio to glucagon in cats as a function of increasing dosage

Cryst. glucagon in first injection (*mcg./Kg.*)	Number of observations	Rise in blood sugar (*mg./100 ml.*)		Response ratio
		First injection Test dose	Second injection Standard*	
0.05	14	19.1 ± 10.22†	49.6 ± 17.2†	0.38 ± 0.040‡
0.10	12	32.0 ± 10.2	45.4 ± 12.0	0.72 ± 0.065
0.20	12	44.5 ± 15.5	30.0 ± 8.9	1.53 ± 0.131
0.30	13	55.0 ± 20.6	26.0 ± 10.1	2.13 ± 0.131
0.40	21	71.0 ± 19.9	24.0 ± 9.6	2.95 ± 0.228
5.0	4	155 ± 22.3	0	Indeterminate
15.0	8	100 ± 13.5	0	Indeterminate

* Glucagon equivalent to 0.2 mcg./Kg. of crystalline preparation.
† Standard deviation.
‡ Standard error of the mean.

differences of the individual animals. Instead of using the net blood sugar rise as a measure for activity, it is advantageous to use the quotient, obtained by dividing the net maximum increase following injection of the sample by the net maximum rise caused by the standard preparation during the second test period. This quotient or *response ratio* improves the accuracy of the test considerably. It can be seen from column 4 in Table II that the standard error of the mean does not exceed 10 per cent for any of the samples investigated. The effect of an appropriate constant dose of the standard preparation depends to some degree on the magnitude of the hyperglycemia produced by the first injection. The broken curve in Figure 1 clearly illustrates the inverse relationship between sample and standard response. The latter decreases to zero as the response to the first injection reaches its maximum following a dose of 1 to 2 mcg. of crystalline

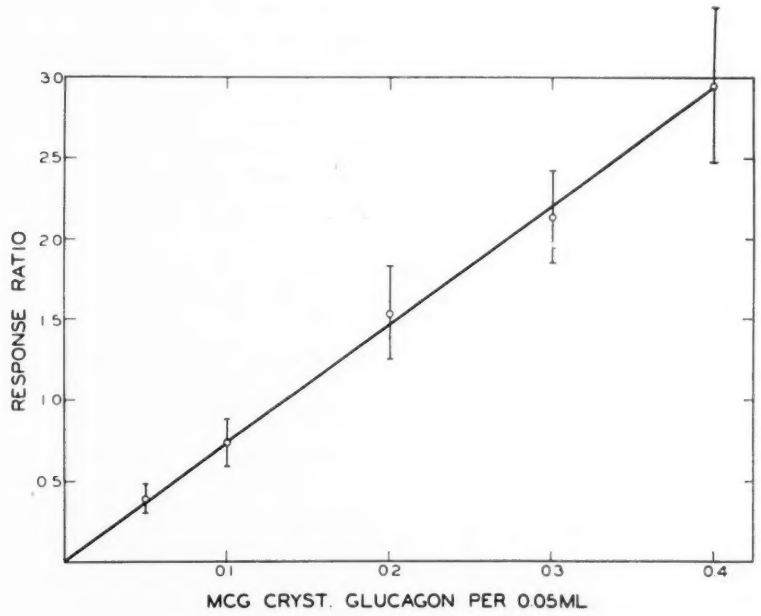

FIG. 2. DOSAGE RESPONSE RATIO CURVE

The standard preparation used was equivalent to a solution containing 4 mcg. of crystalline glucagon per ml.

glucagon per Kg. The numerical values of the quotients, as well as their fiducial limits increase rapidly in the region of maximum hyperglycemia. This region, therefore, is not suitable for measurements of activity. As pointed out earlier, the first response should be limited to 20 to 50 mg. per cent. The dosage-response ratio curve in this range is a linear function as is shown in Figure 2. The slope of the straight line is a function of the potency of the standard preparation. The standard used in these experiments was equivalent to a solution of 4 mcg. of crystalline glucagon per ml.

EXPERIMENTAL PROCEDURE

A number of crystalline insulin preparations have been tested for their content of hyperglycemic factor.

Preparation of samples

Six mg. of crystalline Zn-insulin were dissolved in approximately 2 ml. of dilute acid and the pH was adjusted to 7.5 with 0.2 per cent sodium hydroxide. The volume was brought to 10 ml. after addition of 3.5 ml. of a one per cent cysteine solution of pH 7.5. The mixture was incubated for 16 hours at 18 to 23° C. This incubation period is sufficient to destroy most of the insulin activity.

The response ratios were determined as described above, injecting 0.05 ml. of incubated mixture per Kg. cat body weight. The protein concentration of the samples was adjusted so that the usual dose administered yielded a response ratio close to 0.8 or 1.6. These were the ratios obtained with similarly treated glucagon-free insulin preparations to which 0.3 and 0.6 per cent by weight of crystalline glucagon had been added. The glucagon-free insulin used for these experiments was a special preparation, lot No. 208-158B-238, having a potency of 24.4 μ per mg. It was essentially free of glucagon, since administration of 33 to 600 mcg. per Kg. body weight did not cause any rise in blood sugar.

RESULTS AND DISCUSSION

The analytical data for the different preparations are summarized in Table III. The dose of crystalline glucagon administered by injecting the glucagon-containing reference samples was 0.1 and 0.2 mcg. per Kg. cat body weight, respectively. The response ratios of these samples are, within the limit of error, identical with the ones obtained by administering identical amounts of crystalline glucagon alone. It appears therefore that the other components of the incubation mixture have no effect on the glucagon activity. Hence, use was made of the dosage response ratio curve to compute the glucagon content of samples. Response ratios ranging from 0.7 to 1.7 yield the most reproducible portion of the curve. For this reason only assays with ratios within this range have been considered. The results summarized in Table III indicate that a number of samples of crystalline Zn-insulin have a glucagon content of approximately 0.3 to 0.5 per cent. It seems unlikely that this small quantity can be detected by most physico-chemical methods. Therefore, the presence of glucagon does not offer an adequate explanation for the heterogeneities reported in insulin preparations.

In a recent paper, Root, Ellis, and Staub (17) have presented evidence that addition of glucagon

TABLE III

Determination of glucagon content of crystalline insulin samples

Preparation	mcg. Protein per Kg. cat	Number of animals	Mean response ratio	Fiducial limits $P < 0.05$	% Glucagon in preparation
Glucagon-free insulin + 0.3% glucagon	30	19	0.797 ± 0.055*	0.685–0.909	0.3
Glucagon-free insulin + 0.6% glucagon	30	14	1.65 ± 0.109	1.416–1.884	0.6
Cryst. Zn insulin					
A	30	14	0.741 ± 0.056	0.620–0.862	0.33 ± 0.10†
B	30	15	0.775 ± 0.079	0.607–0.943	0.35 ± 0.11
C	30	18	0.902 ± 0.066	0.764–1.04	0.41 ± 0.11
D	30	18	1.02 ± 0.078	0.887–1.153	0.46 ± 0.13
E	30	18	0.88 ± 0.071	0.731–1.029	0.40 ± 0.11
ML535664	30	14	1.15 ± 0.11	0.914–1.386	0.52 ± 0.15

* Standard error of the mean.

† Obtained from the propagation of error formula for a quotient of two random variables (E. B. Wilson, An Introduction to Scientific Research, McGraw Hill, 1952).

to insulin in quantities up to 50 or 100 per cent by weight were without effect on the assay of insulin by either the mouse or the rabbit methods. Because of the short duration of the hyperglycemia caused by glucagon, its effect has disappeared before the insulin hypoglycemia was well established. Root (18) has found that administration of large amounts of highly purified glucagon over long periods to rats and rabbits does not cause any pathological changes. Furthermore, no permanent nor lasting alteration of the blood sugar level was observed. On the basis of these experiments it appears that glucagon has no significant influence on the hypoglycemic activity of insulin. Similarly there is no evidence that glucagon elicits undesirable side effects.

SUMMARY

The hyperglycemic factor content of some crystalline insulin samples has been determined on a quantitative basis. The crystalline Zn-insulin preparations contained 0.3 to 0.5 per cent of the factor. The effect of glucagon in insulin preparations is discussed.

REFERENCES

1. Kimball, C. P., and Murlin, J. R., Aqueous extracts of pancreas. III. Some precipitation reactions of insulin. J. Biol. Chem., 1923, **58**, 337.
2. Nielsen, N. A., Die Wirkung des Insulins auf den Glykogengehalt der perfundierten kaninchenleber. Biochem. Ztschr., 1931, **230**, 259.
3. Bürger, M., Das Glukagon. Fortschr. diagn. u. therap., 1950, **1**, 225.
4. Conn, J. W., Endocrine regulation of the blood sugar. Ann. Int. Med., 1953, **38**, 179.
5. Staub, A., Sinn, L., and Behrens, O. K., Purification and crystallization of hyperglycemic glycogenolytic factor (HGF). Science, 1953, **117**, 628.
6. Staub, A., Sinn, L., and Behrens, O. K., Characterization of glucagon (hyperglycemic-glycogenolytic factor). Federation Proc., 1954, **13**, 303.
7. Bürger, M., and Brandt, W., Über das Glukagon (Die Hyperglykämisierende Substanz des Pankreas). Ztschr. f. d. ges. exper. Med., 1935, **96**, 375.
8. Fredericq, E., and Neurath, H., The interaction of insulin with thiocyanate and other anions. The minimum molecular weight of insulin. J. Am. Chem. Soc., 1950, **72**, 2684.
9. Harfenist, E. J., and Craig, L. C., Countercurrent distribution studies with insulin. J. Am. Chem. Soc., 1952, **74**, 3083.
10. Sutherland, E. W., Cori, C. F., Haynes, R., and Olsen, N. S., Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. J. Biol. Chem., 1949, **180**, 825.
11. Timasheff, S. N., Brown, R. A., and Kirkwood, J. G., The fractionation of insulin by electrophoresis-convection. J. Am. Chem. Soc., 1953, **75**, 3121.
12. Weitzel, G., Fretzdorff, A. M., Strecker, F. J., and Roester, U., Zinkgehalt und Glukagoneffekt Kristallisierter Insulinpräparate. Ztschr. f. physiol. Chem., 1953, **293**, 190.
13. Porter, R. R., Partition chromatography of insulin and other proteins. Biochem. J., 1953, **53**, 320.
14. Mohnike, G., and Boser, H., Ein Hyperglykämisierend-Glykogenolytisches Pankreasnucleoproteid. Ztschr. f. d. ges. exper. Med., 1954, **123**, 415.
15. Olsen, N. S., and Klein, J. R., Hyperglycemia induced by certain insulin preparations. Proc. Soc. Exper. Biol. & Med., 1947, **66**, 86.
16. Somogyi, M., A reagent for the copper-iodometric determination of very small amounts of sugar. J. Biol. Chem., 1937, **117**, 771.
17. Root, M. A., Ellis, J., and Staub, A., Effect of glucagon on insulin hypoglycemia. Proc. Soc. Exper. Biol. & Med., 1954, **85**, 507.
18. Root, M. A., Proc. Soc. Exper. Biol. & Med., In press.

OBSERVATIONS ON THE INHERITANCE OF SICKLE-CELL HEMOGLOBIN AND HEMOGLOBIN C[1]

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The recognition of hemoglobin C (1, 2) an hereditary abnormality of hemoglobin, occurring in Negroes, has been important for the clinical and genetic concepts of the hereditary hemolytic anemias. The presence of hemoglobin C appears to depend upon a single gene which is transmitted as a simple Mendelian dominant (2–4). The presence of sickle-cell hemoglobin is similarly determined by a single autosomal dominant gene. While the clinical syndrome accompanying the simultaneous presence of sickle and C hemoglobin has been described, the precise relationships between the genes for hemoglobin C and sickle hemoglobin have not yet been elucidated. The possibilities are: 1) That the genes for sickle-cell and hemoglobin C are inherited independently and that the clinical syndrome results from the interaction of non-allelic genes; 2) that the genes for sickle-cell hemoglobin and hemoglobin C are alleles or are closely linked upon the same chromosome. To distinguish between allelism and close linkage would require a very large series. However, by studying the offspring of marriages of sickle-cell-hemoglobin C disease × normal, some conclusions as to independence *vs.* allelism or close linkage may be reached. The present report includes seven families in which both sickle-cell hemoglobin and hemoglobin C have been found, in addition to three similar families which were reported previously (4). The number of observations is now large enough to permit some conclusions about the relationships of the genes for C and S hemoglobins.

Two additional cases of hemoglobin C disease (homozygosity for the gene for hemoglobin C) are presented. The clinical features of the cases of sickle-cell-hemoglobin C disease in this study are similar to those previously reported (2–6). The case histories are presented primarily to provide a documentary basis for the genetic considerations.

[1] This research has been partially supported by a gift of the Blood Transfusion Association.

METHODS

Hemoglobin C and sickle hemoglobin were identified by filter paper electrophoresis (7). Each specimen containing hemoglobin C was placed upon a filter paper containing four or five other samples of this abnormal hemoglobin. During a single run, all specimens of hemoglobin C had the same mobility, thus eliminating the possibility of erroneous identification of hemoglobin E as hemoglobin C. Some of the papers of family studies are reproduced in the photographs in Figure 1. Alkali denaturation was carried out by an adaptation of the method of Lichtman and Watson, and Ponder and Levine (8, 9). Hemoglobin C was crystallized by the method of Drabkin (10); its crystalline characteristics are identical with those of normal adult hemoglobin when examined under the ordinary microscope.

CASE REPORTS

Hemoglobin C disease

I. D. C., a Negro insurance salesman, was born in British Guiana. He was first seen in the out-patient clinic in 1946 at the age of 44 for right lower quadrant pain, of uncertain etiology. There was a history of migratory polyarthritis accompanied by malaise at the age of 22 which had subsided after a few months. At age 34, he had another episode of polyarthritis following gonorrheal urethritis. Physical examination in 1946 revealed a well-developed, well-nourished Negro male, who appeared to be in good health. There was slight cardiac enlargement, with a soft diastolic murmur along the left cardiac border. The blood pressure was 140/90. No enlargement of the liver or spleen was detected. Laboratory findings included hemoglobin of 13.0 grams per cent and ESR 3 millimeters in one hour (Westergren). Both the Kline and the Wassermann tests were strongly positive; no abnormalities were found in the spinal fluid. The patient received a course of penicillin and bismuth. He was next seen in 1950 for symptoms of a duodenal ulcer which was demonstrated on gastrointestinal x-rays. In 1952, he was admitted to the hospital for gastrointestinal bleeding. At that time, the spleen was palpable 2 to 3 cm. below the left costal margin. The aortic diastolic murmur was apparently un-

changed from previous description. Laboratory findings included hemoglobin 9.7 grams per cent, stool guaiac test was strongly positive. Many target cells were noted on stained smears, and a sickling preparation was negative. The bleeding subsided promptly and the patient was discharged from the hospital. In 1953, he was readmitted for repair of an inguinal hernia. The splenomegaly was still present; the diastolic cardiac murmur was not heard by some observers. Laboratory findings at this time were: Hemoglobin 13.5 grams per cent, WBC 5,300 per cu. mm., with a normal differential count. Reticulocytes were 1.6 per cent, platelets 144,000 per cu. mm. The blood smear showed nearly 100 per cent target cells. Bone marrow aspiration revealed erythroid hyperplasia; 62 per cent of the nucleated cells were of the erythroid series. Mechanical fragility was 3 per cent (normal less than 5 per cent). Osmotic fragility: hemolysis began at 0.40, incomplete at 0.20 for patient's erythrocytes with range of 0.45 to 0.30 per cent saline for normal control. Four weeks following the uneventful herniorraphy he was seen in clinic where the hemoglobin was 13.6 grams per cent. Erythrocytes were 4.4 million per cu.mm. with 1.9 per cent reticulocytes. Hematocrit was 37 per cent.

Filter paper electrophoresis showed that all the hemoglobin was of the C variety. His wife was found to have normal hemoglobin, and their only son had hemoglobin C trait, the combination of C and normal hemoglobin (Figure 2, group II).

II. Ja. T., a three and one-half-year-old Negro child, was seen as a member of family J. G. (Figure 2, group IV). His mother (V. T.) had been told that she was anemic and was found to have sickle-cell-hemoglobin C disease. His father (Jo. T.) had always been well; blood smears taken during this study showed an increased number of target cells and the filter paper electrophoresis of the father's hemoglobin showed the combination of C and normal hemoglobin. The son, Ja. T., was said to have been jaundiced at birth, with persistence of jaundice for 2 to 3 months thereafter. The child had been slow in development, and at age three and one-half years could not stand alone or talk, although he could apparently hear normally. At the time these studies were done he was hospitalized at a rehabilitation hospital with a diagnosis of cerebral palsy of the choreoathetotic type. Physical examination revealed retardation in development; there was no jaundice and the spleen was not felt.

The laboratory findings included: Hemoglobin 12 grams per cent, erythrocytes 4.5 million per cu. mm.; hematocrit was 40 per cent with reticulocytes 1.2 per cent. The leucocyte count was 8,100 per cu. mm., neutrophils 26, lymphocytes 72, monocytes 2. Target cells comprised over 85 per cent of the erythrocytes.

Filter paper electrophoresis revealed an unusual pattern (see Figure 1): The major part of the hemoglobin was in the position of hemoglobin C. However, there was a small amount of another hemoglobin which on repeated and longer runs migrated between sickle and normal hemoglobin. This is the position of migration of fetal hemoglobin. Alkali denaturation showed 6 per cent alkali-resistant hemoglobin (values for normal hemoglobin by this method are below 3 per cent [8]). The faster component on the paper electrophoresis pattern, probably fetal hemoglobin, comprised 14 to 16 per cent of the total hemoglobin as determined by dye elution (6).

Family studies: Group I, Marriage of sickle-cell trait individuals × hemoglobin C trait individuals

Family R. L. (Figure 2, group I, c). R. L., Jr. was seen initially in 1947 at the age of 11, for dyspnea of one year's duration. A febrile illness with vomiting, chest and abdominal pain during the preceding year had been diagnosed as rheumatic fever. He had cardiac enlargement by physical examination and by x-ray with systolic and diastolic apical murmurs. The spleen was not felt. Laboratory findings were: Hemoglobin 8.2 grams per cent, erythrocytes, 3.7 million per cu. mm. Sickling tests were positive, with many target cells noted on smear. Electrocardiogram was within normal limits. Patient was followed during the next seven years in cardiac and hematology clinics. He developed a leg ulcer in 1951 which healed slowly. In 1952, he developed abdominal pain with signs of peritoneal irritation in the right lower quadrant; a normal appendix was removed. During recent years he has had no more abdominal pain; his hemoglobin has ranged between 10 and 12 grams per cent with 2 to 3 per cent reticulocytes and many target cells have always been found on stained smears. Whether he has rheumatic heart disease in addition to his hemolytic anemia has not been established.

Filter paper electrophoresis showed that his hemoglobin was a mixture of C and sickle hemoglobin. Additional members of the family were studied (Figure 1), one younger brother also had sickle-cell-hemoglobin C disease.

The brother (H. L.) was seen in the out-patient department in 1952 at the age of 8, for frequent attacks of pharyngitis. Physical examination revealed the spleen to be palpable 3 to 4 cm. below the left costal margin. The hemoglobin was 9.4 grams per cent, erythrocytes 3.7 million per cu. mm. Leucocytes and platelets were normal. Many target cells were noted on stained smears. The patient was seen subsequently in the clinic for an attack of abdominal pain, and on another occasion for pain in the forearm; both of these episodes subsided within a few days. The splenomegaly did not change appreciably during the two years of observation. Filter paper electrophoresis of the hemoglobin revealed the combination of sickle and C hemoglobin.

Of the other siblings, two have sickle-cell trait, three have hemoglobin C trait, and one died in the neonatal period. None of the offspring studied was normal.

Family A. B. (Figure 2, group I, a). A. B., an 11-year-old Negro schoolgirl, was first seen in the Babies' Hospital in 1953 for pain in the left elbow of three days' duration. She had had recurrent attacks of polyarthritis since the age of six. Physical examination revealed tenderness, without swelling, of the right elbow. No cardiac abnormalities were noted. The spleen tip was

palpable just below the left costal margin. Laboratory examinations showed: Hemoglobin, 9.9 grams per cent; erythrocytes, 3.9 million per cu. mm.; hematocrit 33.7 per cent. Target cells were noted in descriptions of blood smears. ESR was 4 mm. in one hour (Westergren). Serum bilirubin was 3.5 milligrams per cent. The pain subsided without significant decrease in hemoglobin and the patient was discharged from the hospital.

Filter paper electrophoresis showed her hemoglobin to consist of a mixture of sickle and C hemoglobin. The hemoglobin of her sister showed the pattern of sickle-cell trait. The mother had hemoglobin C trait and the father sickle-cell trait.

Family J. G. (Figure 2, group I, d). J. G., the index case in this family, was admitted to an Air Force hospital for subacute bacterial endocarditis. There was a history of "aching" of the extremities at the age of six, which had been treated with several months of bed rest. One year prior to hospitalization the patient had been rejected as a blood donor because of anemia. Physical examination revealed a basal diastolic murmur, and the spleen was palpable 2 to 3 cm. below the left costal margin. Laboratory findings were: Hemoglobin 11.5 grams per cent, erythrocytes 4.9 million per cu. mm., hematocrit 34 per cent, reticulocytes 2.5 per cent. The leucocytes were normal. An increased number of target cells was noted on blood smears and sickling preparations were positive. Filter paper electrophoresis of the patient's hemoglobin revealed the characteristic pattern of sickle-cell-hemoglobin C disease.

The remainder of the family were studied: The father had sickle-cell trait, the mother, hemoglobin C trait. Of the five siblings tested (one was unavailable) three had a pattern identical with that of the patient; one was normal, and one had sickle-cell trait.

Clinical and hematological data on the three siblings in this family with sickle-cell-hemoglobin C disease were incomplete. However, V. T. had a history of anemia and arthralgias with a positive sickling preparation, and an increased number of target cells on stained blood smears. Her reticulocytes were 3.4 per cent. She was the mother of Ja. T. (hemoglobin C disease). One sister, Je. G., age 26, had symptoms of weakness and arthralgias with a hemoglobin of 12 grams per cent, hematocrit of

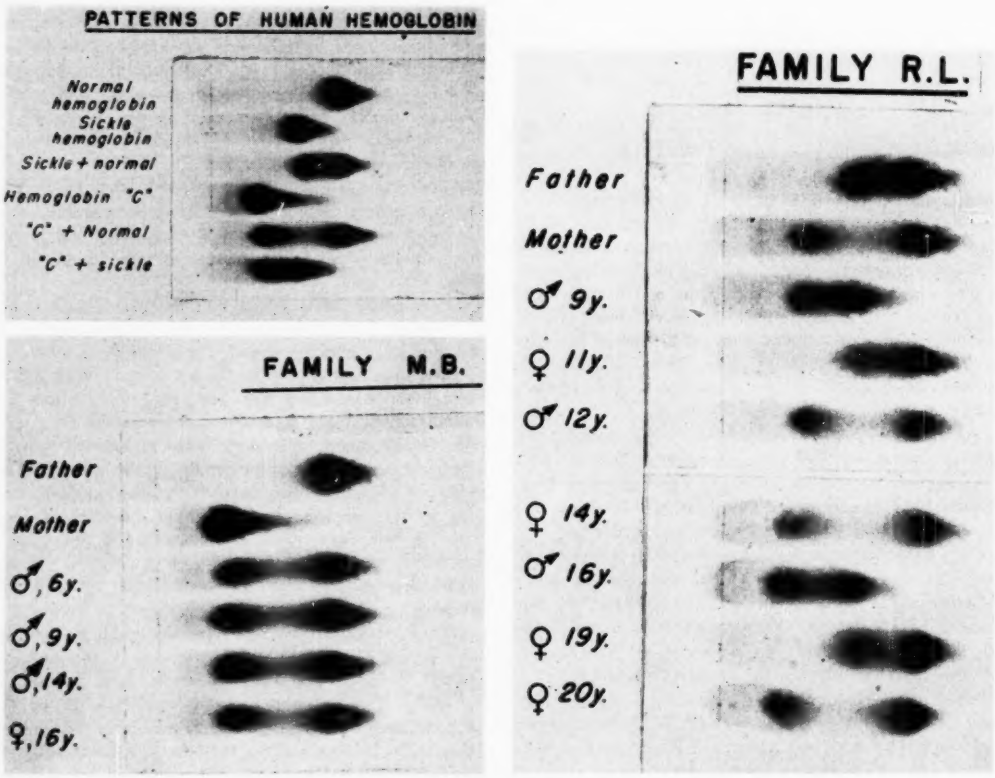

FIG. 1. PHOTOGRAPHS OF THE FILTER PAPER PREPARATIONS OF SELECTED FAMILY STUDIES

The distortion of the hemoglobin pattern of the mother of Family C. W. is the result of prolonged storage of that hemoglobin specimen.

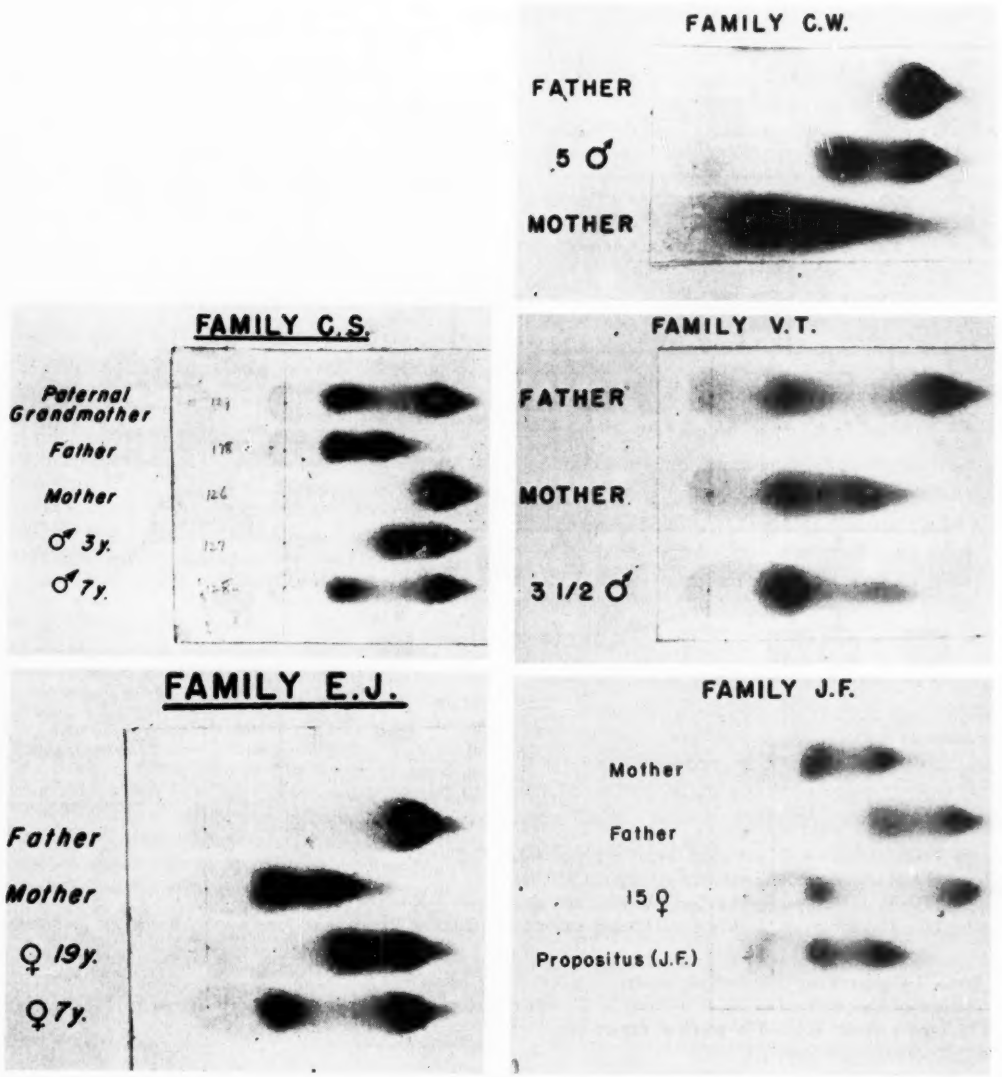

FIG. 1—*Continued*

36 per cent and about 30 per cent target cells on smears. Her brother, A. G., age 17, was asymptomatic, but his spleen was palpable 3 cm. below the left costal margin. His hemoglobin was 14 grams per cent, the hematocrit was 37 per cent and a blood smear showed 50 per cent target cells. Specimens of hemoglobin from these three siblings (V. T., Je. G., and A. G.) all showed a mixture of C and sickle hemoglobin.

Group II. Marriage of hemoglobin C disease × normal (*Figure 2, group II, a and b*). M. B., homozygous for the gene for hemoglobin C, previously reported (4) and her husband whose hemoglobin was normal, had four children, all with hemoglobin C trait (Figure 1). D. C. (clinical history reported above) and his wife whose hemoglobin was normal have one son with hemoglobin C trait.

Group III. Marriage of sickle-cell-hemoglobin C disease × normal

Family E. J. (*Figure 2, group III, c*). E. S. J. was first seen in 1932 at the age of 22 during her second pregnancy. She had her initial attack of arthralgias and fever at the age of 12, and had had several attacks

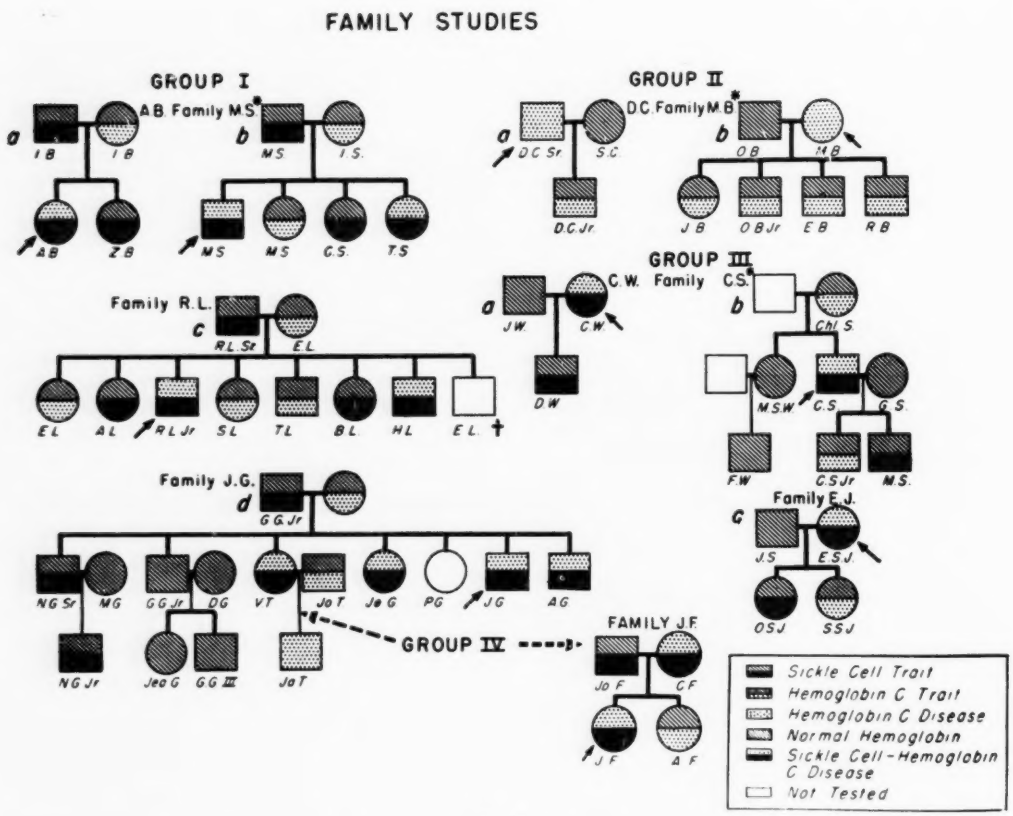

FIG. 2.

GROUP I—MARRIAGE OF SICKLE-CELL TRAIT × HEMOGLOBIN C TRAIT
GROUP II—MARRIAGE OF HEMOGLOBIN C DISEASE × NORMAL
GROUP III—MARRIAGE OF SICKLE-CELL-HEMOGLOBIN C DISEASE × NORMAL
GROUP IV—MARRIAGE OF SICKLE-CELL-HEMOGLOBIN C DISEASE × INDIVIDUAL HETEROZYGOUS FOR AN ABNORMAL HEMOGLOBIN

Arrow indicates index case in each family.
The sex of two children of M. B. (Group II b) was erroneous in the diagram in earlier report (4).
(*) Family study included in previous report (4).

thereafter. Her first pregnancy was terminated because of anemia and "heart trouble." In 1932, systolic and diastolic apical murmurs, mild cardiac enlargement, and a spleen palpable 3 to 4 cm. below the left costal margin were noted. A sickling preparation was found to be positive, the hemoglobin was "75 per cent," erythrocytes 3.7 million per cu. mm. with 14 per cent reticulocytes. The pregnancy was uneventful and a healthy child was delivered at term. Thereafter this patient was admitted to the hospital several times: in 1938 and in 1951, early therapeutic abortions were done and in 1944 a second normal pregnancy proceeded to term uneventfully. Two hospitalizations for pleuritic pain, without roentgenographic findings, may have been related to sickle-cell disease; the level of hemoglobin did not change during these admissions, however. For 22 years of observation the splenomegaly remained unchanged, as did the cardiac size and cardiac murmurs. The hemoglobin ranged from 10 to 13 grams per cent with reticulocytes varying between 4 and 14 per cent. Target cells were noted repeatedly on stained blood smears. The hemoglobin of the patient was found to consist of a mixture of sickle and C hemoglobins; the hemoglobin of her first husband J. S. (the father of both children) was normal. One child had sickle-cell trait: the other, hemoglobin C trait.

Family C. W. (*Figure 2, group III, a*). The clinical findings of C. W., a 30-year-old Negro housewife with

sickle-cell-hemoglobin C disease, were reported previously (4). Hemoglobin studies were subsequently carried out on the husband and son of C. W. The hemoglobin of her husband proved to be normal; their only son had the combination of sickle and normal hemoglobin.

Family C. S. (Figure 2, group III, b). This family consisting of a father with sickle-cell-hemoglobin C disease has been reported previously (4). The hemoglobin of his wife was normal; one son had sickle-cell trait, and the other hemoglobin C trait.

Group IV. Marriage of sickle-cell-hemoglobin C disease × the heterozygous state for an abnormal hemoglobin.

A. Sickle-cell hemoglobin C disease × sickle cell trait.

Family J. F. (Figure 2, group IV). J. F., a Negro schoolgirl, had her first symptoms at age 10 when she was hospitalized for fever and pains in the extremities. A diagnosis of poliomyelitis was made, but no residual deformities occurred. A similar attack occurred one year later, again without sequelae. At the age of 13 she was admitted to the Presbyterian Hospital for right lower quadrant pain and vomiting. The hemoglobin was 12.4 grams per cent, latent sickling was noted in a wet preparation. The leucocyte count was 16,100 per cu. mm. with 92 per cent neutrophils. A normal appendix was removed. The patient had no further symptoms until the age of 17, when she had an episode of right arm pain which subsided after a few days. Hemoglobin was then 10.3 grams per cent, erythrocytes were 3 million per cu. mm. Reticulocytes were 2.9 per cent with rare nucleated red blood cells and many target cells on peripheral blood smears. Filter paper electrophoresis revealed her hemoglobin to be a mixture of sickle and C hemoglobin. The hemoglobin of her sister was shown to be that of hemoglobin C trait (Figure 1). The hemoglobin of her mother was found to be identical, by filter paper electrophoresis, with that of the patient. After some delay, a hemoglobin specimen was obtained from the father; he was shown to have sickle-cell trait. Blood grouping studies (including ABO, MN, Rh, Kell, and Duffy) were carried out on the members of this family; no evidence of non-paternity was found.

C. W. F., the mother, was 37 years of age. She had a history of anemia with pregnancies, and an episode of unexplained hematuria at the age of 28. At the time of the study, her spleen was palpable 3 to 4 cm. below the left costal margin. Her hemoglobin was 12.5 grams per cent, erythrocytes were 4.5 million per cu. mm. Leucocytes were 4,600 per cu. mm. and reticulocytes were 2.4 per cent.

B. Marriage of sickle-cell-hemoglobin C disease × hemoglobin C trait

(Figure 2, group IV). This marriage (of V. T. and Jo. T.) was encountered in Family J. G. The only son had hemoglobin C disease.

RESULTS

The data from this and previous study (4) are summarized in Table I. Photographs of the filter paper electrophoresis study of one family in each of groups I and II, and of each family study in groups III and IV are shown in Figure 1.

DISCUSSION

The data from groups I, II, III, and IV indicate that the genes for C and sickle hemoglobin probably segregate as simple Mendelian dominants. It is noteworthy that an unexpectedly small number of normal offspring of marriage C/+ × S/+ were found. Even if the four index cases (C/S) are excluded, there is still an apparent deficiency of offspring with normal hemoglobin. The total number of children is, however, too small to permit reliable statistical evaluation, and furthermore this has not been noted in a series observed by

TABLE I

	Total marriages	Number of offspring					Total offspring
		S/+	C/+	+/+	C/S	C/C	
Group I							
Marriage S/+ × C/+*	4	5	4	1	9		19
Group II							
Marriage C/C × +/+	2		5				5
Group III							
Marriage C/S × +/+	3	3	2				5
Group IV							
A Marriage C/S × S/+	1		1		1		2
B Marriage C/S × C/+	1					1	1

* + indicates corresponding normal allele, *i.e.*, S/+ = sickle-cell trait; C/+ = hemoglobin C trait; +/+ = all normal hemoglobin.

others (3). Should this relative increase in abnormal offspring reappear in larger series, further investigation (for example of the possibility of maternal effect) would be necessary.

The data from group III, marriage C/S × +/+, are important in establishing the relationship of the genes for C and S hemoglobin. This group includes five offspring of three such marriages; none of the offspring is of either parental type with respect to hemoglobin.

This evidence, while not eliminating the possibility that the genes for C and S hemoglobin are independent, is statistically in favor of allelism or close linkage. If the genes for C and S hemoglobin were actually independent, one would expect to find among five such offspring some who had inherited both abnormal genes from the affected parent, or neither gene.

Further support for the allelic nature of S and C is derived from study of the offspring of the marriages in group IV. The only marriage, C/S × C/+, produced a child of genotype C/C; this is an observation entirely consistent with the theory that C and S are allelic or closely linked.

The observation in Family J. F., where a parent of type C/S had a child of type C/S is consistent with allelism since the father's genotype was S/+. Consequently, the offspring J. F. would have inherited the gene for sickle-cell hemoglobin from the father and the gene for C hemoglobin from her mother. The nature of the hemoglobin of the sibling A. F. (C/+) was also consistent with allelism or close linkage.

While these studies provide good evidence against independence of the genes for sickle-cell and C hemoglobin, they do not serve to distinguish between allelism and close linkage of these genes. For the distinction between allelism and close linkage, by statistical methods, a very large series would be required. Although final proof of the allelic nature of the two genes is not available, certain data may be interpreted in the light of physiological genetics as supporting the allelic theory. If the genes for these abnormal hemoglobins were either closely linked, or on different chromosomes, then in the case of the simultaneous presence of C and S hemoglobins, each gene should be accompanied by a corresponding normal allele. If independent, the arrangement might be depicted thus:

$$\frac{C}{+}\ \frac{S}{+};\quad \text{if closely linked}\quad \frac{C\ +}{+\ S}$$

In either of these situations, the individual with genes for both C and S hemoglobins might be expected to have in addition to C and S hemoglobin, a significant amount of normal hemoglobin. Actually the examination in this laboratory of specimens of hemoglobin from 14 different individuals with sickle-cell-hemoglobin C disease has failed to reveal any hemoglobin component which could be definitely identified as normal adult hemoglobin. The majority of the specimens contained less than 5 per cent of hemoglobin migrating on filter paper faster than sickle hemoglobin; all contained less than 10 per cent of such hemoglobin. Furthermore, if filter paper electrophoresis was allowed to proceed for a longer period, this faster component of hemoglobin migrated to a position consistent with the migration of fetal hemoglobin (between sickle and normal adult hemoglobin).

The assumption could be made that the simultaneous presence of the genes for C and S hemoglobin in a single individual suppresses the formation of normal adult hemoglobin. This speculation does not find support in the study of the heterozygotes S/+ or C/+, where normal adult hemoglobin comprises 50 per cent or more of the total hemoglobin. These considerations add support to the hypothesis that the genes for C and S hemoglobins are alleles.

The findings in the two cases of hemoglobin C disease included in this report are similar to previous descriptions (4, 11, 12) of this disorder. In both of these patients, target cells comprised 85 per cent or more of the erythrocytes; D. C. (age 52 years) had splenomegaly, while Ja. T. (age three and one-half years) did not. The presence of 15 per cent of fetal hemoglobin in patient Ja. T. whose hemoglobin level was 12 grams per cent indicates that factors other than anemia must control the synthesis of fetal hemoglobin.

SUMMARY

Observations on a total of ten families in which C and S hemoglobin occurred indicate that the genes for sickle and C hemoglobin segregate as simple Mendelian dominants.

Five offspring of three marriages of individuals

with sickle-cell-hemoglobin C disease to individuals with normal hemoglobin were found to have either sickle-cell trait or hemoglobin C trait. This supports the hypothesis that the genes for C and sickle hemoglobin are alleles.

Observations on three offspring of individuals with sickle-cell-hemoglobin C disease and marital partners heterozygous for an abnormal hemoglobin were consistent with allelism of the genes for sickle and C hemoglobin.

The fact that individuals with sickle-cell-hemoglobin C disease do not have any appreciable amount of normal adult hemoglobin is interpreted as evidence favoring allelism of the genes rather than linkage or independence.

Two additional cases of hemoglobin C disease, *i.e.*, homozygosity for the gene for hemoglobin C, were described.

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REFERENCES

1. Itano, H. A., and Neel, J. V., A new inherited abnormality of human hemoglobin. Proc. Nat. Acad. Sc., 1950, **36**, 613.
2. Kaplan, E., Zuelzer, W. W., and Neel, J. V., A new inherited abnormality of hemoglobin and its interaction with sickle cell hemoglobin. Blood, 1951, **6**, 1240.
3. Neel, J. V., Kaplan, E., and Zuelzer, W. W., Further studies on hemoglobin C. I. A description of three additional families segregating for hemoglobin C and sickle-cell-hemoglobin. Blood, 1953, **8**, 724.
4. Ranney, H. M., Larson, D. L., and McCormack, G. H., Jr., Some clinical, biochemical and genetic observations on hemoglobin C. J. Clin. Invest., 1953, **32**, 1277.
5. Smith, E. W., and Conley, C. L., Filter paper electrophoresis of human hemoglobins with special reference to the incidence and clinical significance of hemoglobin C. Bull. Johns Hopkins Hosp., 1953, **93**, 94.
6. Kaplan, E., Zuelzer, W. W., and Neel, J. V., Further studies on hemoglobin C. II. The hematologic effects of hemoglobin C alone and in combination with sickle cell hemoglobin. Blood, 1953, **8**, 735.
7. Larson, D. L., and Ranney, H. M., Filter paper electrophoresis of human hemoglobin. J. Clin. Invest., 1953, **32**, 1070.
8. Lichtman, H. C., and Watson, R. J., Personal communication.
9. Ponder, E., and Levine, P., Fetal and adult hemoglobins in the blood of infants affected with hemolytic disease of the newborn. Blood, 1949, **4**, 1264.
10. Drabkin, D. L., Spectrophotometric studies. XV. Hydration of macro sized crystals of human hemoglobin, and osmotic concentrations in red cells. J. Biol. Chem., 1950, **185**, 231.
11. Spaet, T. H., Alway, R. H., and Ward, G., Homozygous type "C" hemoglobin. Pediatrics, 1953, **12**, 483.
12. Watson, R. J., The hereditary anemias. Bull. New York Acad. Med., 1954, **30**, 106.

PLASMA 17-KETOSTEROIDS OF FULL-TERM AND PREMATURE INFANTS [1, 2]

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The adrenal cortex of the newborn exhibits a relative lack of responsiveness to the administration of adrenocorticotrophic hormone which has been the subject of several investigations and is adequately reviewed elsewhere (1). These observations, together with the presence of the fetal reticular zone of the adrenal cortex and the recent report of very low concentrations of compound F-like substances in newborn plasma (2), are all reminiscent of the findings in infants and children with congenital adrenal hyperplasia (3, 4).

Since children with congenital adrenal hyperplasia have been shown to have markedly elevated concentrations of plasma neutral 17-ketosteroids (5), it was thought desirable to investigate the concentrations of these steroids in the plasma of newborn infants. The purpose of this paper is to describe analytic data obtained when plasma samples from a group of normal full-term infants and a group of premature infants were analyzed for neutral 17-ketosteroids.

METHODS

Normal full-term infants in the newborn nursery and premature infants in the premature unit of the Syracuse Memorial Hospital were utilized for the study. Seventeen analyses on plasma from 19 full-term newborns were done (plasma was pooled in two instances). Twenty analyses were done on as many premature infants. Venous blood was collected by femoral puncture and oxalated. Plasma was separated by centrifugation at about 2500 r.p.m. for 10 minutes and stored at 0° F. until analyzed. Neutral 17-ketosteroids were determined on 3 to 7 ml. samples of plasma as described elsewhere (6). The results were expressed as μg. per 100 ml. of plasma. This method utilizes chromatography on "Florisil" columns, the Zimmermann reaction being carried out on the residue of a chloroform eluate. The latter solvent is used, since crystalline 17-ketosteroids are eluted from the column by it, but not by ethanol-chloroform mixtures When plasma samples from premature infants 9, 10, and 12 were analyzed (Table I), the Zimmermann chromogen was eluted by pure chloroform, but not by 2 per cent ethanol in chloroform. This chromatographic distribution was consistent with that obtained using crystalline 17-ketosteroids.

Since hyperbilirubinemia is not infrequent in the newborn period, a 10 mg. sample of crystalline bilirubin was subjected to the entire analytical procedure. No measurable Zimmermann chromogen was found, indicating that elevated serum bilirubin concentrations should not cause falsely high values for plasma 17-ketosteroids.

RESULTS

Studies on full-term newborns

Measurement of plasma neutral 17-ketosteroids on samples from full-term infants during the first five days of life are shown in Figure 1. For purposes of comparison the transverse dotted line at 56 μg. per 100 ml. shows the mean value (± standard error of the mean) for plasma 17-ketosteroid concentration in non-pregnant women (6). The data indicate that the full-term newborn may have plasma neutral 17-ketosteroid values during the first 48 hours of life which are in excess of those found in the adult female. There is a rapid fall of plasma neutral 17-ketosteroid values during the first five days of life to very low concentrations by the fifth day. No measurable plasma values were obtained in normal full-term infants after the first week of life. Analyses done on samples from older infants and pre-adolescent children indicated that plasma 17-ketosteroids, if present, were in concentrations lower than this method could estimate.

Studies on premature infants

Table I provides data on 20 premature infants, age 1 to 42 days and with body weights of 793 to

[1] Presented at the annual meeting of the American Pediatric Society, Atlantic City, New Jersey, May 7, 1953.

[2] This study was supported in part by a grant from the Division of Research Grants, National Institutes of Health, Department of Health, Education and Welfare.

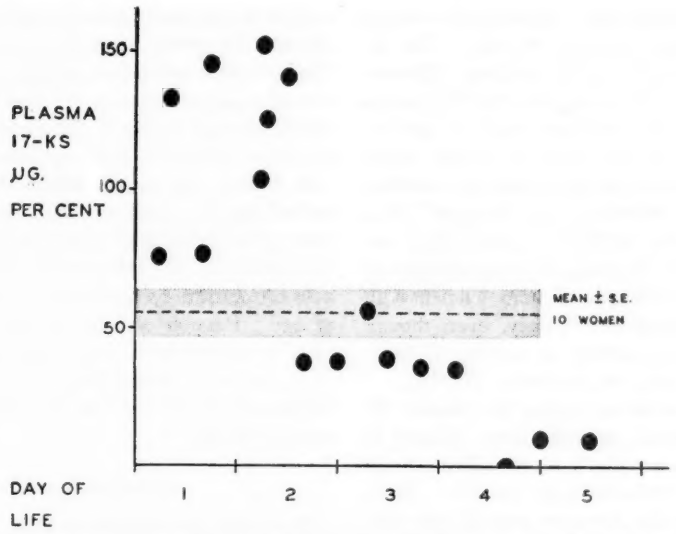

FIG. 1. PLASMA NEUTRAL 17-KETOSTEROIDS DURING THE FIRST FIVE DAYS OF LIFE IN NORMAL, FULL-TERM INFANTS

2381 gm. The value of 533 μg. 17-ketosteroids per 100 ml. of plasma in Patient 1 is the highest concentration thus far obtained on a human subject in this laboratory. Measurable concentrations of plasma 17-ketosteroids were found in all the premature infants examined.

TABLE I

Plasma 17-ketosteroid data on premature infants of various body weights and ages

Patient No.	Sex	Age *days*	Body weight: At birth *gm.*	Body weight: Attained wt. *gm.*	Plasma 17-KS *μg. per 100 ml.*
1	M	1	1,134	1,134	533
2	F	1	793	793	181
3	F	2	1,871	1,757	183
4	F	2	1,843	—	106
5	M	2	1,871	—	105
6	F	2	2,154	—	67
7	M	3	1,615	—	310
8	M	4	2,097	1,984	36
9	M	8	1,049	964	76
10	M	8	1,615	1,361	8
11	F	13	1,984	1,928	32
12	F	15	1,899	1,984	22
13	F	16	2,041	2,381	7
14	M	17	1,729	1,729	105
15	F	17	1,672	1,928	49
16	M	18	1,956	1,871	62
17	M	27	1,331	1,729	19
18	F	32	1,361	1,956	42
19	F	34	1,700	2,268	34
20	F	42	1,446	2,069	40

DISCUSSION

The present finding in full-term infants of elevated plasma 17-ketosteroid concentrations in the first days of life with subsequent fall, fits in with the results obtained by Read, Venning, and Ripstein on urinary 17-ketosteroids in the newborn (7). These workers found a sharp diminution in the excretion of urinary 17-ketosteroids during the first nine days of life. Whereas the plasma 17-ketosteroid values in the full-term newborns of the present study are somewhat higher than the values found in adult females, the urinary excretion of 17-ketosteroids in the newborn was found by Read, Venning, and Ripstein (7) and also by Klein (8) to represent only a small fraction of the adult figure. This disparity between plasma and urine may be the result of an endogenous "load" of 17-ketosteroids presented to the kidneys of the newborn infant for urinary excretion.

The possible sources of the elevated plasma 17-ketosteroids in the newborn infant represent an interesting enigma. Recent findings which may throw light on this problem are the observations that the 17-ketosteroid concentration is consistently greater in umbilical cord plasma than in simultaneously collected maternal plasma and that women near the end of pregnancy have plasma 17-

ketosteroid concentrations significantly lower than do non-pregnant women (9, 10). The direction of the concentration gradient between cord and maternal plasma suggests that the plasma 17-ketosteroids of the newborn may be derived from some tissue in the fetal circulation other than from the mother, possibly from the internal cortex of the fetal adrenal or the placenta. It is relevant that several previous workers have observed a progressive increase during pregnancy of urinary 17-ketosteroid excretion in women with proven adrenocortical insufficiency, even though these women had essentially no urinary 17-ketosteroid excretion prior to pregnancy (11–13).

The high and persistent values for plasma 17-ketosteroids obtained on premature infants in the present study are much more difficult to fit into any known endocrinologic scheme. Since values for glomerular filtration rate in the premature infant are lower than in the full-term newborn, it is not too surprising that the highest plasma 17-ketosteroid values of the neonatal period were obtained in premature infants. On the other hand it is hard to see how this mechanism could be invoked to explain elevated plasma 17-ketosteroid values in premature infants four or five weeks old, unless there were a continuous endogenous source of plasma 17-ketosteroids. It would be tempting to suggest that delayed involution of the internal cortex of the fetal adrenal is responsible for this phenomenon in the premature infant. However, Lewis and Pappenheimer (14) and Potter (15) state generally that premature infants show no delay of involution of the internal adrenal cortex. In contradistinction Moeri presents specific protocols of premature infants showing retardation of internal zone involution (16). The latter observation would go along nicely with the finding of persistent elevation of plasma 17-ketosteroid concentration in the premature infant. Since the histological studies referred to are in disagreement, it would appear that judgment on this point should be reserved until our state of knowledge is more satisfactory.

SUMMARY

Plasma samples have been analyzed for neutral 17-ketosteroids from 19 full-term, newborn infants, and from 20 premature infants. The premature infants had attained weights ranging from 793 to 2381 grams when the samples were taken. The full-term newborns showed plasma 17-ketosteroid concentrations in the first 48 hours of life which were in excess of the mean value for non-pregnant women. These plasma concentrations then rapidly fell to unmeasurable values by the end of the first week of life. The premature infants showed maximal values higher than the values shown by the full-term infants, and measurable concentrations were still found up to 42 days of age. Possible sources of plasma 17-ketosteroids in the newborn were discussed. There appears to be as yet no adequate explanation for the differences found between the full-term and premature infants.

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REFERENCES

1. Klein, R., Some aspects of adrenal physiology in the newborn period and in early infancy *in* Proceedings of the Second Clinical ACTH Conference, J. R. Mote, Ed., New York, The Blakiston Co., 1951, vol. I, p. 123.
2. Klein, R., Fortunato, J., and Papadatos, C., Free blood corticoids in the newborn infant. J. Clin. Invest., 1954, **33**, 35.
3. Blackman, S. S., Jr., Concerning the function and origin of the reticular zone of the adrenal cortex. Hyperplasia in the adrenogenital syndrome. Bull. Johns Hopkins Hosp., 1946, **78**, 180.
4. Kelley, V. C., Ely, R. S., and Raile, R. B., Metabolic studies in patients with congenital adrenal hyperplasia. Effects of cortisone therapy. J. Clin. Endocrinol. & Metab., 1952, **12**, 1140.
5. Gardner, L. I., Increased plasma 17-ketosteroid concentration in congenital adrenal hyperplasia. Proc. Soc. Exper. Biol. & Med., 1953, **83**, 251.
6. Gardner, L. I., Plasma neutral 17-ketosteroids. I. Technique of estimation. J. Clin. Endocrinol. & Metab., 1953, **13**, 941.
7. Read, C. H., Venning, E. H., and Ripstein, M. P., Adrenal cortical function in newly-born infants. J. Clin. Endocrinol., 1950, **10**, 845.
8. Klein, R., Adrenocortical control of sodium and potassium excretion in the newborn period. J. Clin. Invest., 1951, **30**, 318.
9. Gardner, L. I., and Walton, R. L., Plasma 17-ketosteroids of the human fetus: Demonstration of concentration gradient between cord and maternal circulations. Helvet. paediat. acta, 1954, **9**, fasc. 4–6.

10. Gardner, L. I., Walton, R. L., Ellis, W., and Hughes, E. C., Diminished plasma 17-ketosteroid concentration in pregnancy. Proc. Soc. Exper. Biol. & Med., 1954, **86**, 804.
11. Samuels, L. T., Evans, G. T., and McKelvey, J. L., Ovarian and placental function in Addison's disease. Endocrinology, 1943, **32**, 422.
12. Knowlton, A. I., Mudge, G. H., and Jailer, J. W., Pregnancy in Addison's disease. Report of four patients. J. Clin. Endocrinol., 1949, **9**, 514.
13. Jailer, J. W., and Knowlton, A. I., Simulated adrenocortical activity during pregnancy in an Addisonian patient. J. Clin. Invest., 1950, **29**, 1430.
14. Lewis, R. W., and Pappenheimer, A. M., A study of the involutional changes which occur in the adrenal cortex during infancy. J. Med. Research, 1916, **34**, 81.
15. Potter, E. L., Pathology of the Fetus and the Newborn. Chicago, The Year Book Publishers, 1952, p. 282.
16. Moeri, E., Les surrénales chez le foetus, le nouveau-né, le nourrisson et l'enfant. Rapports avec l'hypophyse. Signification et involution de la corticale foetale. Lacunes de la corticale externe. Surrénales de l'anencèphale. Acta endocrinol., 1951, **8**, 259.

STUDIES ON MYOCARDIAL METABOLISM. III. CORONARY BLOOD FLOW, MYOCARDIAL OXYGEN CONSUMPTION AND CARBOHYDRATE METABOLISM IN EXPERIMENTAL HEMORRHAGIC SHOCK [1, 2]

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A series of reports have been published on the cause of irreversibility from hemorrhagic shock (1–5). Wiggers and Werle (2, 3), on the basis of pressure and volume curves obtained from dogs during various phases of hemorrhagic shock, suggested the possibility that the deterioration of myocardial expulsive power contributed to circulatory failure and that this myocardial depression contributed to the reappearance of circulatory failure during the normovolemic phase. It was assumed that the trigger mechanism for myocardial depression was a decrease in the coronary flow with resulting myocardial ischemia. Opdyke and Foreman (6) indicated that during the period of hypotension, the actual coronary flow was seriously curtailed, providing ample opportunity for myocardial changes. Immediately following reinfusion, however, the coronary flow appeared adequate.

Several studies have indicated the existence of general tissue anoxia in shock (7, 8). Thus, anoxia manifests itself in shock in a state of metabolic acidosis, which is at least partially the result of an increase in blood lactate (9, 10). Simultaneously, the blood levels of pyruvate and glucose increase (11, 12). A shift toward anaerobic metabolism in impending or progressive shock is further suggested by an increase in the ratio of lactate to pyruvate in the blood (11). The studies of McShan, Potter, Goldman, Shipley, and Meyer (11) suggested that a steady progressive depletion of high energy phosphate compounds and of glycogen occurs during the impending stage of traumatic shock. Greig and Govier in studying the changes in intermediary metabolism in hemorrhagic shock found a decrease in the cocarboxylase content of tissues of dogs in shock and in anoxic anoxia (13). These authors believed that under these conditions cocarboxylase is dephosphorylated, resulting in a breakdown of cocarboxylase into thiamin and phosphate. This appears to be in line with the work of Ochoa who found in *in vitro* work that in many tissues under anaerobic conditions cocarboxylase is enzymatically destroyed (14). Greig also found changes in cozymase (D.P.N.) in muscle liver and brains after hemorrhage and De Turk and Greig reported diminished ability of tissues to metabolize amino acids (15, 16).

This report deals with a study of changes in coronary flow and myocardial oxygen consumption in experimental hemorrhagic shock and with the effect of these changes on the myocardial metabolism of glucose, pyruvate, and lactate.

MATERIAL AND METHODS

The experiments were carried out on a total of 28 dogs anesthetized intravenously with sodium pentobarbital (30 mg. per Kg. weight). A tracheotomy tube was inserted for collection of expired air and to permit the animal to inhale nitrous oxide. A femoral artery was cannulated for withdrawal of arterial blood samples and for the measurement of mean arterial pressures with a mercury manometer. In experiments in which cardiac output as well as coronary flow were determined, two No. 7 birdseye cardiac catheters were inserted into the heart through a single jugular vein. Under fluoroscopic guidance, one was then placed in the pulmonary artery, the other in the coronary sinus. In experiments in which the cardiac output was not determined, a single catheter was introduced in the coronary sinus. All animals were heparinized with 400 units of heparin per Kg. weight, intravenously, with an additional 500 units being given every 30 minutes.

During the control period the dog's mean arterial pressure and the pulse rate were recorded and simultaneous

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samples of coronary vein and femoral arterial blood were collected for the determination of blood concentrations of oxygen, pyruvate, glucose, and lactate. Following this, expired air was collected in a Douglas bag over a time interval of from 3 to 6 minutes. During the collection of expired air, arterial and pulmonary arterial blood were collected. This was followed by determination of coronary blood flow using the nitrous oxide desaturation method previously described (17, 18).

Irreversible hemorrhagic shock was produced by a modification of the method of Wiggers (1). The main difference between the procedure of Wiggers and that employed in this study was to extend the critical period of lowest blood pressure (30 mm. Hg) to 60 minutes. In line with the procedure of Wiggers, oligemic shock was produced as follows: Blood was withdrawn from the femoral artery at the rate of approximately 50 cc. per minute until mean systemic arterial pressures had fallen to 50 mm. Hg. Arterial pressures were then maintained at this level for 90 minutes by withdrawal of small quantities of blood at varying intervals. Following this, the pressure was further lowered to 30 mm. Hg and maintained for 60 minutes at this level by small extractions or infusions of blood.

At the end of the critical period (60 minutes) samples of arterial and coronary vein blood were drawn for analysis of metabolites and the coronary blood flow was again determined. Following this, the normovolemic phase of shock was produced by reinfusing the filtered blood withdrawn previously. In addition, 200 cc. of blood were infused as replacement of blood removed for analytical purposes. After from 15 to 20 minutes to allow equilibration, blood samples were again drawn and the coronary flow was determined. It was assumed that these animals were in the compensatory stage of normovolemic shock.

In order to follow changes in cardiac metabolism at more frequent intervals, three experiments were performed in which blood samples were drawn at 30-minute intervals. Only glucose, lactate, pyruvate, and ketone bodies were determined in these samples and the coronary flow was not measured.

Blood was collected in lightly oiled syringes to which 8 drops of heparin had been added to prevent clotting. Samples were analyzed for oxygen and nitrous oxide on the manometric apparatus of Van Slyke and Neill (19). Carbon dioxide and oxygen in expired air were measured in the Haldane apparatus (20). Blood glucose was determined with the method of Hagedorn and Jensen, using the method of Somogyi to prepare the blood filtrates (21, 22). Pyruvate was determined according to the method of Friedemann and Haugen, using a trichloracetic acid filtrate (23). Lactate was measured with the method of Barker and Summerson (24). Ketones were determined with a modification of the micromethod of Greenberg and Lester (25).

The coronary blood flow per 100 gm. was calculated according to the method previously described (17, 26). Blood flow through the left ventricular muscle was obtained by multiplying blood flow per 100 gm. times left ventricular weight. This was determined by the formula of Herrmann (27) (body weight in Kg. $\times$ 0.0037). Myocardial usage of oxygen and of various foodstuffs was obtained as the product of coronary flow per 100 gm. or per left ventricular weight times the respective myocardial oxygen, glucose, lactate or pyruvate extractions. Cardiac work and mechanical efficiency of the left ventricle were calculated as previously described (17).

For the statistical work-up of the data, paired algebraic differences were recorded for each animal and in each classification for which usable data were available. In each case the "student" t test was applied to test the null hypothesis that the mean difference was zero

$$\left(t = \frac{(\text{mean } \sqrt{n-1})}{\text{std. deviation}}\right) \text{ (28).}$$

The probability value "p" was obtained from tables printed elsewhere (28). Sigma expresses the standard deviation and p the probability value.

RESULTS

Hemodynamic studies during oligemic shock

Table I illustrates that the cardiac output declined during the oligemic phase. The average fall was 3443 cc. with a standard deviation of 2311.2 cc. The fall was significant at the 5 per cent level (p of 0.025). The stroke volume also diminished an average of 22 cc. per beat with a standard deviation of 16.69 cc. and a p of 0.025. Both these changes were statistically significant. The large variations of the cardiac output during the control period are probably the result of anesthesia. The fall in cardiac output and stroke volume was primarily a reflection of increased systemic oxygen extraction since the oxygen consumption was not altered. This "stagnant anoxemia" has been repeatedly observed by a series of investigators (29, 30). The rise in systemic arteriovenous oxygen difference was statistically highly significant (p value of 0.005). No significant alterations occurred in the heart rate during hemorrhagic shock (p value of 0.2).

In contrast to the systemic oxygen extraction, the myocardial oxygen extraction showed no significant change during oligemic shock (mean fall of 1.00 vol. per cent sigma of 1.81 and a probability of 0.15). This is in line with the previous observation that changes in myocardial oxygen consumption are brought about by variations in coronary flow and not by alterations in myocardial oxygen extraction (31, 32). The coronary flow

TABLE I

No.	Weight	Card. output cc.	Heart rate/ min.	St. vol./cc.	Mean B.P. mm./Hg	Systemic O_2 ext. vol. %	Cor. O_2 ext. vol. %	L.V. work Kg./met.	L.V. wt. gm.	Cor. resist. mm. Hg/ 100 gm./ min.	Hema-tocrit %	Cor. flow cc./100 gm./min.	Cor. flow L.V. cc./min.	Myoc. O_2 us. cc./min./ 100 gm.	L.V. O_2 usage cc./min.	En. cost Kg./met.	Effi-ciency %
S1A*	13.6	4,860	180	27.0	140	1.7	12.1	9.24	50	.85	—	165	82	20.0	9.9	19.8	46.5
B		760	160	4.8	50	11.1	11.4	.52	50	.41	—	121	60.5	13.8	6.9	13.8	3.7
S2A	19.9	7,090	160	44.3	138	2.6	9.2	13.3	74	.70	—	196	145	18.0	13.3	26.6	50.0
B		860	212	4.1	50	12.0	12.2	.58	74	.83	—	60	44	7.3	5.4	10.8	5.3
S3A	12.8	7,200	140	51.4	140	1.1	9.8	13.7	47	1.1	—	138	60.5	13.7	5.9	11.8	—
B		682	212	3.2	50	9.7	10.8	.46	47	.71	—	70	33	7.6	3.5	7.0	6.6
S4A	15.8	2,110	172	12.3	115	3.5	11.0	3.30	58	.91	—	127	74	14.0	8.1	16.2	20.3
B		428	160	2.7	50	10.3	10.6	.29	58	.60	—	84	49	8.9	5.2	10.4	2.8
S5A	12.6	2,145	160	13.4	170	7.9	12.6	4.96	46	1.15	—	148	68	18.7	8.6	17.2	28.7
C		1,940	130	14.9	90	4.7	10.5	2.40	46	.64	—	142	65	14.9	6.8	13.6	17.7
S7A	16.2	4,270	192	22.2	160	3.5	12.6	9.28	60	.62	—	259	155	32.7	19.5	39.0	23.8
C		3,250	200	16.2	160	6.3	14.6	7.08	60	.71	—	226	136	33.0	19.8	39.6	21.5
S8A	22.0	2,340	148	15.8	140	5.0	14.1	4.46	81	1.19	—	118	96	16.6	13.5	27.0	26.7
C		1,430	104	13.8	115	7.7	10.3	2.24	81	1.35	—	85	69	8.8	7.1	14.2	25.4
S9A	14.6	1,662	140	11.9	110	6.7	15.9	2.49	54	2.20	—	50	27	7.95	4.3	8.6	34.5
C		703	160	4.4	85	13.0	15.3	.81	54	1.55	—	54.5	29	8.30	4.4	8.8	9.8
S12A	16.1	2,500	168	14.9	135	3.4	11.4	4.59	59	.98	44	137	81	15.6	9.2	18.4	25.0
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S12C		1,340	140	9.6	145	5.9	14.3	2.69	59	1.78	48	82	48	11.6	6.9	13.8	19.2
S13A	18.3	4,300	144	29.8	140	2.15	10.25	8.20	68	.85	38	165	112	16.9	11.5	23.0	35.6
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C		1,580	144	11.0	120	4.6	10.5	2.58	68	.81	38	148	101	15.6	10.6	21.2	12.1
S14A	13.6	2,700	126	21.4	125	4.0	12.4	4.59	50	1.10	41	108	54	13.4	6.7	13.4	34.2
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C		800	138	5.8	105	10.9	12.1	1.14	50	1.48	49	71	35	8.6	4.24	8.48	13.5

* A = Control
B = Oligemic shock
C = Normovolemic shock

TABLE I—*Continued*

No.	Weight	Card. output *cc.*	Heart rate/ min.	St. vol./cc.	Mean B.P. *mm./Hg*	Systemic O_2 ext. *vol. %*	Cor. O_2 ext. *vol. %*	L.V. work *Kg./met.*	L.V. wt. *gm.*	Cor. resist. *mm. Hg/ 100 gm./ min.*	Hema-tocrit %	Cor. flow *cc./100 gm./min.*	Cor. flow L.V. *cc./min.*	Myoc. O_2 us. *cc./min./ 100 gm.*	L.V. O_2 usage *cc./min.*	En. cost *Kg./met.*	Effi-ciency %
S15A	14.2	1,810	144	12.6	120	4.6	10.7	2.95	52	.81	32	149	78	15.9	8.3	16.6	17.8
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C		1,450	104	17.8	120	5.9	11.7	2.37	52	1.05	45	114	59	13.3	6.9	13.8	17.2
S16A	13.6	2,420	174	13.9	140	3.7	10.7	4.61	50	.58	35	240	120	25.7	12.8	25.7	14.0
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C		2,310	132	17.5	140	4.7	10.2	4.40	50	1.27	45	111	55	11.3	5.6	11.2	39.2
S17A	13.5	1,935	12.8	15.1	125	4.2	10.9	3.37	50	1.04	38	120	60	13.1	6.5	13.1	25.7
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C		1,325	9.6	13.8	136	5.3	9.1	2.46	50	1.27	51	107	53	9.7	4.8	9.6	39.2
S19A	13.0	2,100	180	11.7	145	4.3	7.2	4.15	48	.67	42	215	103	15.50	7.4	14.8	35.7
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C		970	164	5.9	130	7.7	8.5	2.17	48	2.28	57	57	27	4.85	2.3	46	4.72
S20A	13.0	1,435	180	8.0	140	4.8	9.8	2.73	48	.98	—	142	68	13.9	6.6	13.2	20.7
B		306	160	1.9	35	13.6	13.6	.15	48	.55	—	64	31	8.7	4.2	8.4	1.8
S21A	13.9	1,430	160	8.9	140	6.8	14.5	2.72	52	1.01	—	138	72	20.7	10.4	20.8	12.5
B		427	160	2.6	35	14.1	13.8	.21	52	.64	—	55	29	7.5	4.0	8.0	3.8
S22A	18.0	3,750	168	22.3	125	2.9	10.2	6.39	67	1.04	43	120	80	12.2	8.2	16.4	39.0
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C		1,170	148	7.9	120	9.5	15.9	1.91	67	1.58	53	76	51	120	8.1	16.2	11.8
S23A	20.8	2,060	160	12.9	145	5.3	13.5	4.06	77	1.36	40	107	82	14.5	11.1	22.2	18.3
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C		1,320	160	8.3	155	6.8	15.7	2.79	77	1.81	55	86	66	13.5	10.4	20.8	13.4
S24A	13.7	1,250	140	8.9	155	5.5	13.3	2.64	51	.74	41	210	108	28.0	14.4	28.8	9.2
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C		1,210	134	9.0	80	5.4	10.1	1.32	51	.71	52	112	57	11.3	5.8	11.6	8.8
S25A	14.8	1,210	200	6.1	135	6.2	14.8	2.22	55	1.06	43	127	70	18.8	10.4	20.8	10.7
B	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C		750	180	4.2	120	11.4	15.8	1.22	55	1.79	60	67	37	10.6	5.9	11.8	10.3

and, therefore, myocardial oxygen usage decreased significantly during oligemic shock (mean fall in oxygen usage of 7.75 cc. with a sigma of 3.08 and a probability of 0.005). In contrast to the systemic circulation, a decrease in coronary flow produced no change in myocardial oxygen extraction.

Changes in left ventricular work and myocardial efficiency observed during oligemic shock are illustrated in Table I and Figure 1. As left ventricular work decreased proportionally more than myocardial oxygen usage, the myocardial efficiency declined. The mean percentage fall in efficiency was − 26.5 per cent with a sigma of 14.5.

The coronary resistance declined during oligemic shock (mean of − 0.302 with a probability of 0.01). This was probably the result of myocardial anoxia which is known to be an effective coronary vasodilator.

Hemodynamic changes during normovolemic shock

Many of the changes observed during the oligemic phase persisted after reinfusion of blood. For example, both cardiac output and stroke volume remained considerably below their control values (decrease in the mean of − 993 cc. and − 4.85 and a sigma of 798.8 and of 6.72, respectively). The probability values were 0.005 and 0.01, respectively. The systemic arteriovenous oxygen difference remained elevated (Table I) (mean of 3.12 with probability of 0.005 and a sigma of 2.11). Although there was considerable rise in systemic blood pressure from levels recorded during oligemia, both coronary flow and myocardial oxygen consumption were still significantly below their control levels (mean difference of − 49 cc. of − 4.9 cc. and sigma of 44.3 and 5.93, respectively) (Table I). This differ-

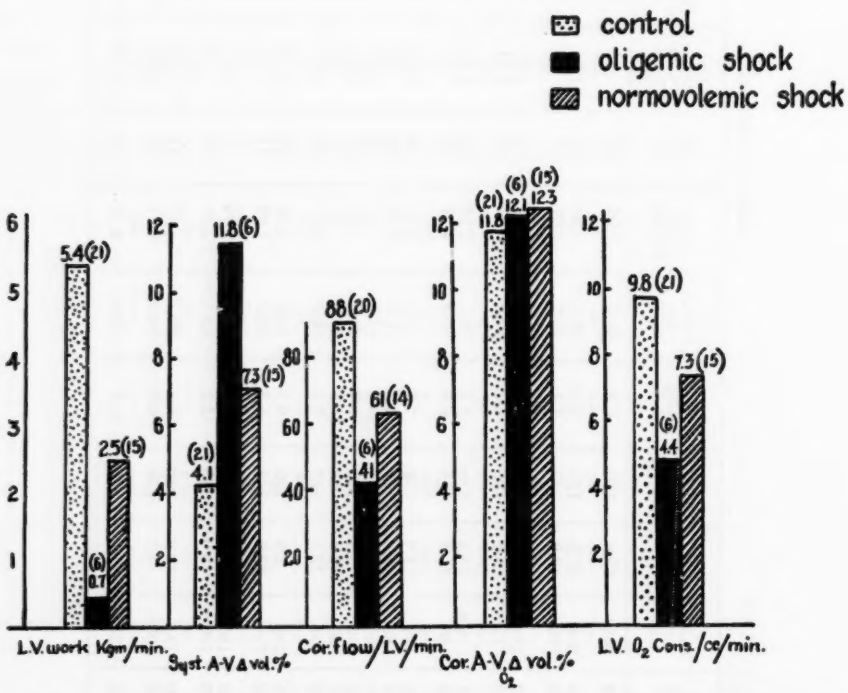

FIG. 1. THE HEMODYNAMIC CHANGES OCCURRING DURING NORMOVOLEMIC AND OLIGEMIC SHOCK

It may be seen that with exception of the coronary arteriovenous oxygen extraction which remains unchanged during the oligemic and normovolemic phase, the left ventricular work, systemic oxygen content, the coronary flow, and the left ventricular oxygen consumption remain below control values during the normovolemic phase.

TABLE II

No.	Glucose			Lactate			Pyruvate		
	Art. concen. *Mgm. %*	Myoc. extra. *Mgm. %*	L.V. usage *Mgm./min.*	Art. concen. *Mgm. %*	Myoc. extra. *Mgm. %*	L.V. usage *Mgm./min.*	Art. concen. *Mgm. %*	Myoc. extra. *Mgm. %*	L.V. usage *Mgm./min.*
S1A	94.3	3.7	3.07	12.9	4.6	3.77	2.1	.85	.70
B	153.0	2.8	1.69	108.0	3.2	1.94	4.2	−.5	−.31
S2A	93.8	0	0	20.0	7.4	10.7	2.5	1.10	1.60
B	206	0	0	38.6	4.9	2.2	5.1	−.69	−.31
S3A	86.1	5.6	3.39	12.2	5.1	3.01	2.3	.96	.59
B	543.0	5.0	1.65	81.3	18.3	6.04	3.4	−.48	−.15
S4A	87.3	2.3	1.70	7.3	1.4	1.04	1.4	.2	.15
B	131.0	13.5	6.62	97.0	6.0	2.94	6.0	−.8	−.24
S5A	94.0	3.5	2.38	10.9	3.7	2.52	1.9	.49	.33
C	83.4	5.0	3.25	113.7	2.1	1.37	4.1	−.49	−.32
S7A	91.5	.5	.78	21.6	9.0	14.0	.12	.06	.09
C	110.1	3.2	4.35	89.5	11.5	15.6	.09	.01	.01
S8A	94.6	1.9	1.82	6.9	2.1	2.01	.16	.02	.02
C	46.7	6.5	4.48	127.5	6.5	4.48	.97	.19	.13
S9A	85.7	1.7	.46	28.1	15.8	4.27	.35	.17	.05
C	213.2	.5	.15	86.4	19.9	5.77	1.10	.26	.07
S12A	92.1	1.6	1.30	5.3	2.5	2.05	.34	.02	.02
B	175.1	7.6	—	132.0	15.5	—	1.8	−.68	—
C	93.6	—	—	125.0	13.5	6.48	2.18	−.41	−.20
S13A	98.0	1.7	1.91	15.9	6.0	6.72	.15	.12	.13
B	411.0	1.0	—	110.0	7.0	—	.35	−.05	—
C	150.1	−2.7	−2.73	83.5	6.5	6.57	.42	.06	.06
S14A	92.7	9.0	4.86	18.2	8.2	4.43	.42	.12	.06
B	97.6	−4.0	—	132.2	10.0	—	1.64	−.60	—
C	177.5	−1.5	−5.25	137.2	14.7	5.14	2.16	−.49	−.17
S15A	96.1	1.8	1.40	12.8	3.4	2.65	.33	.05	.04
B	347.0	10.0	—	106.0	11.0	—	1.5	−.85	—
C	145.0	−3.5	−2.06	106.8	15.1	8.90	1.34	−.13	−.07
S16A	75.1	.8	.96	19.4	4.1	4.92	.53	.04	.05
B	203.9	.3	—	136.5	5.0	—	1.5	−.70	—
C	105.0	−.8	−.44	115.0	1.0	.55	1.7	−.05	−.03
S17A	107.8	1.0	.60	12.5	6.3	3.78	.53	.10	.06
B	73.0	1.8	—	117.0	7.5	—	1.30	−.40	—
C	49.8	2.4	1.27	96.5	11.5	6.10	1.20	−.18	−.09
S19A	101.5	3.1	3.2	10.7	2.0	2.06	.47	.03	.03
B	80.8	3.8	—	85.0	14.1	—	1.0	−.10	—
C	—	—	—	58.9	3.2	.85	1.0	−.35	−.09
S20A	2.0	2.0	1.36	17.2	3.7	2.52	.5	.0	—
B	324	11.0	3.41	178.0	12.5	3.88	1.4	−.85	−.26
S21A	130.8	7.0	5.04	23.0	7.4	5.33	.58	.05	.04
B	343.5	3.5	1.02	182.0	12.5	3.63	2.4	−.63	−.18
S22A	102.0	4.0	3.20	13.9	7.1	5.68	.42	—	—
B	226.2	—	—	73.0	11.7	—	1.5	.22	—
C	109.5	2.5	2.81	53.0	11.0	5.62	1.5	.55	.26
S23A	96.3	1.5	1.23	18.9	3.9	3.20	.55	—	—
B	203	2.0	—	115.0	25.0	—	.96	−.25	—
C	94.0	1.8	1.19	107.5	18.0	11.9	1.4	−.56	−.37
S24A	79.0	3.6	3.89	16.0	5.5	5.94	.44	.05	.05
B	—	—	—	—	—	—	—	—	—
C	30.4	−1.6	−.91	110.2	11.5	6.55	1.20	−.25	−.14
S25A	84.3	6.7	4.69	16.7	6.2	4.34	.65	.07	.05
B	181.5	14.5	—	117.5	34.0	—	.88	.13	—
C	123.5	—	—	88.5	26.0	9.63	.57	—	—

ence was statistically significant (p values of 0.005). The effect of retransfusion of blood on myocardial efficiency was not as uniform. Table I illustrates that left ventricular efficiency fell significantly in eight animals, rose in two and showed only a slight change in the remainder of the animals. Because of this wide scatter there was no significant statistical change (p value of 0.050).

Changes in coronary resistance during normovolemic shock were not uniform. Table I illustrates that the coronary resistance rose above control levels in ten experiments, fell in four and showed no significant change in the remainder. Because of the wide scatter the increase in algebraic difference from the control value was just significant at the 5 per cent level (p of 0.025).

RESULTS OF METABOLIC STUDIES

Oligemic shock

The experimental results are summarized in Table II. There was a statistically highly significant increase in the arterial concentrations of glucose, lactate, and pyruvate during oligemic shock (mean rise of 141, 98 and 1.31 mg. per cent respectively with a sigma of 132, 34 and 1.06, respectively and a probability of 0.005 for all three foodstuffs). Similar observations were made by previous investigators (9–12). It is likely that the metabolic acidosis produced by the increase in arterial lactate was the result of tissue hypoxia.

It has been previously demonstrated that the extraction of glucose by the heart muscle depends on its arterial concentration (33, 34). The data in Table II illustrate that although the rise in arterial glucose concentration often exceeded 300 per cent, the rise in myocardial glucose extraction was small and statistically not significant (mean rise of 1.67, sigma of 5.85, and probability of 0.15) (Figure 2). There was even a slight fall in the mean average value for myocardial glucose usage during oligemic shock (mean fall of -0.028, sigma of 2.87, and probability of .05) (Table II). In five experiments the glucose concentration in coronary vein blood exceeded that of arterial blood, the difference ranging from + .8 to 4.0 mg. per cent of glucose (Table II and Figure 2). These results suggested that the heart in oligemic shock has partially lost its ability to utilize glucose.

In contrast, a rise in arterial lactate concentration was accompanied by a statistically significant increase in myocardial lactate extraction (mean rise of 7.43, sigma of 8.07, and a probability of 0.005 [Table II, Figure 3]). However, because of the accompanying fall in coronary flow, the

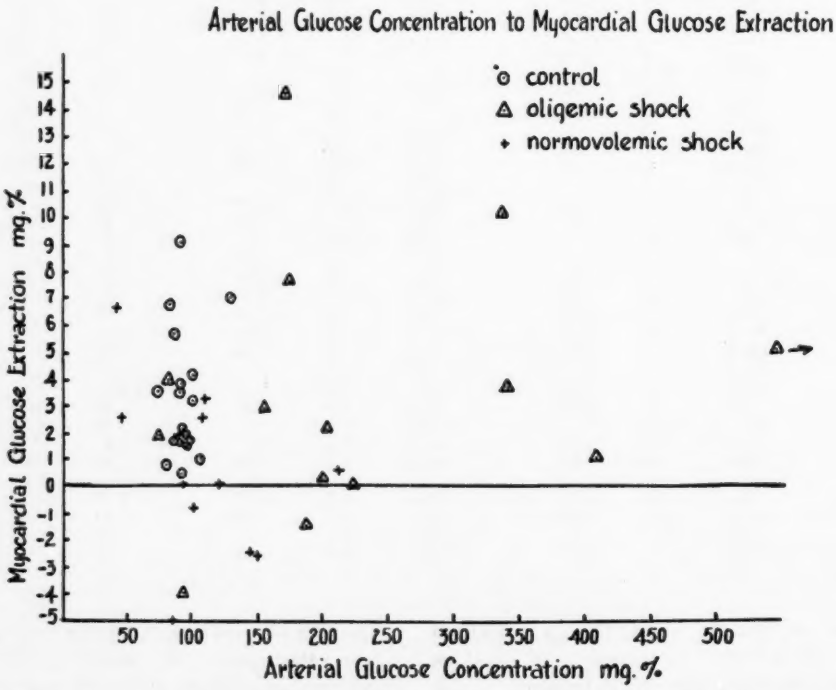

FIG. 2. CHANGES IN ARTERIAL GLUCOSE CONCENTRATION AND MYOCARDIAL GLUCOSE EXTRACTION DURING OLIGEMIC AND NORMOVOLEMIC SHOCK

During oligemic shock the arterial glucose concentration is elevated returning to almost control values during normovolemia. Despite elevated arterial glucose concentration the myocardial glucose extraction is not significantly increased; in some instances glucose concentrations in coronary vein blood even exceed those in arterial blood.

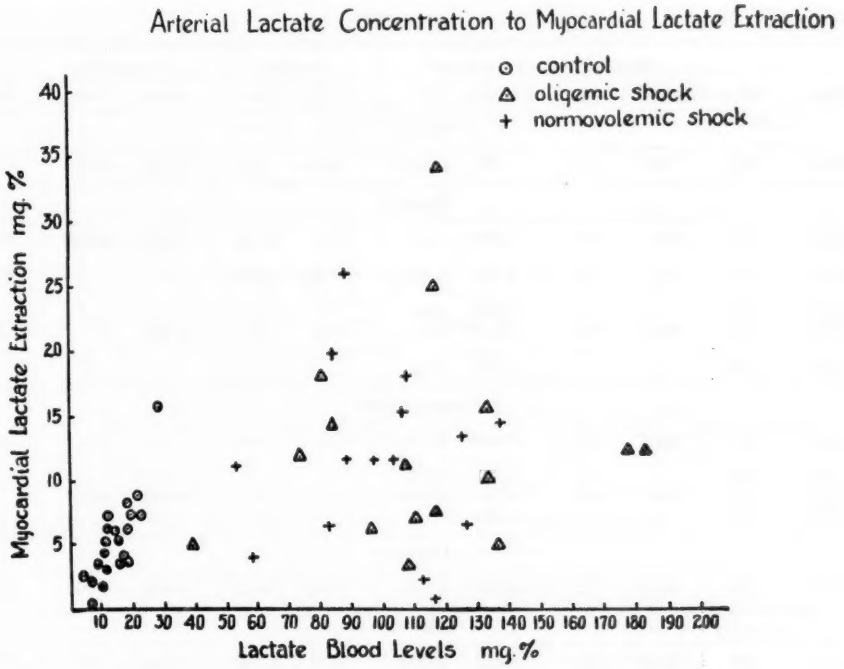

FIG. 3. THE RELATIONSHIP OF ARTERIAL CONCENTRATIONS OF LACTATE TO MYOCARDIAL LACTATE EXTRACTION

Marked elevations in blood lactic acid levels are found in oligemic and normovolemic shock and myocardial lactate extractions are elevated. The lactate concentrations in coronary vein blood never exceed those in arterial blood.

myocardial usage of lactate was not increased (mean fall of -0.39, sigma of 3.92, probability of $\pm .4$). In no instance did the lactate concentration in coronary vein blood exceed that in arterial blood (Table II, Figure 3).

The changes in myocardial pyruvate extraction during oligemic shock are illustrated in Table II and Figure 4. The trend toward a negative myocardial balance of pyruvate is even more conspicuous than in the case of glucose. The pyruvate level in coronary vein blood exceeded that of arterial blood in most instances (Table II and Figure 4). The results were statistically highly significant (the mean fall from the control value was -0.799 mg. per cent with a standard deviation of 0.495 and a probability of 0.005). In contrast to the results of Govier and Greer (35) who found very little change in pyruvate level of arterial blood during oligemic shock, the rise of pyruvate concentration in arterial blood was highly significant (mean rise of 1.32, sigma of 1.06, and a probability value of 0.005). As a result of the negative pyruvate extraction, myocardial usage of pyruvate also became negative (Table II). The difference from the control level was statistically significant at the 5 per cent level (p of 0.025).

Metabolic changes during normovolemic shock

In some instances the trend of metabolic changes, apparent during the oligemic phase, persisted during normovolemic shock. The blood levels of pyruvate and lactate remained significantly elevated above control values (Table II, Figures 3 and 4). (In case of pyruvate a rise of the mean pyruvate level above control of .915 mg. per cent, a probability of 0.005, and a sigma of .602; in the case of lactate, a rise of mean arterial concentration of 84.7 mg. per cent, with a sigma of 24.7, and a probability of 0.005.) In contrast, arterial glucose concentration returned to almost

TABLE III

No.	Time	B.P.	Glucose		Pyruvate		Lactate		Ketone bodies		L/P ratio	% Change
			A. level	Ext.	A. level	Ext.	A. level	Ext.	A. level	Ext.		
						Control						
26	10:15	146	93.4	1.3	.912	.304	9.35	3.42	0.742	−0.07	10.2	0
						Oligemic						
	10:45	50	174	8.8	.589	−.72	35.4	10.00	1.038	0.098	59.6	49.4
												+485
	11:15	50	198	1.8	1.632	1.005	39.22	10.04	—	—	24.5	14.3
												+140
	11:45	50	—	—	2.210	0.05	—	—	—	—	—	
	12:15	30	344.2	3.2	2.440	−0.01	82.0	8.8	1.924	0	33.6	23.4
												+228
	12:45	30	—	—	2.730	0.22	—	—	—	—	—	
						Normovolemic						
	1:30	120	160.8	2.7	2.410	0.39	53.6	9.36	—	—	22.4	12.2
												+220
	2:00	120	—	—	1.362	0.15	—	—	—	—	—	
	2:30	114	120	1.9	1.511	0.419	—	—	0.092	0.077	—	
	3:00	117	—	—	1.026	0	—	—	—	—	—	
						Control						
27	10:15	125	83.6	1.2	0.269	0.054	5.70	0.97	0.936	0.102	21.2	0
						Oligemic						
	11:10	50	163.6	−1.9	1.612	0.392	52.55	12.70	1.146	.00	32.6	11.4
												+54
	11:40	50	106.2	0.2	1.200	0	—	—	—	—	—	
	12:10	50	71.1	1.6	1.705	−0.09	—	—	—	—	—	
	12:40	30	34.	0.2	1.720	−0.310	88.35	10.35	1.288	0.676	51.4	30.2
												+143
	1:10	30	21.5	1.9	1.775	−0.585	—	—	—	—	—	
						Normovolemic						
	1:30	50	25.	1.99	2.280	−0.410	99.5	8.0	0.919	0.184	43.5	22.3
												+105
						Control						
28	10:45	140	83.7	3.7	0.905	0.40	15.8	6.72	.33	0.094	17.6	0
						Oligemic						
	11:30	50	115.2	6.2	1.701	0.431	—	—	—	—	—	
	12:00	50	138	3.4	2.465	0.535	47.6	16.2	0.613	±0	19.4	1.8
												+10.2
	12:30	50	126.3	5.3	2.435	0.594	63.2	22.2	0.800	.235	26.0	8.4
												+19.4
	1:00	30	77.0	−14.	2.200	− .0	—	—	—	—	—	
	1:30	30	56.9	1.5	2.650	−0.340	—	—	0.877	0.219	36.0	18.4
												+104
						Normovolemic						
	2:00	110	46.0	−3.1	2.505	−.325	109.0	15.5	1.042	0.275	43.5	25.9
												+148
	2:30	105	40.8	0.2	2.505	−.290	92.	12.0	0.603	−0.164	37.0	19.4
												+11
	3:00	50	43.4	0.9	3.400	0.120	—	—	—	—	—	
	3:30	80	37.5	−0.1	2.280	0.811	68.7	23.4	1.042	0.329	30.1	12.5
												+7.1
	4:00	70	40.6	3.6	2.521	0.126	—	—	—	—	—	
	4:30	58	—	—	2.921	0.146	49.6	10.8	0.823	0.056	17.0	.6
												−.3

control values (p value of 0.15) making the change from control figures statistically insignificant. Similar observations have been made by other investigators (36). It seemed likely that hyperglycemia during oligemic shock was the result of glycogenolysis with depletion of liver glycogen while the fall in blood glucose during the impending or progressive stages was caused by depletion of liver glycogen (36).

The myocardial extraction of pyruvate remained reduced (Table II and Figure 4) (mean of − 0.258, sigma of 0.303, probability of 0.01). This decline in pyruvate extraction from control level during normovolemia was statistically significant at the 5 per cent level. In some experiments, the pyruvate concentration of coronary vein blood still exceeded that in arterial blood (Table II). Apparently, the infusion of blood had in some instances, failed to correct the metabolic disturbance, initiated during the oligemic phase.

The myocardial extraction of lactate which had risen during the oligemic phase remained significantly elevated after retransfusion of blood (mean rise of 5.75 mg. per cent of lactate with a sigma of 5.94 and a probability value of 0.005). As during the oligemic phase, myocardial lactate usage showed no significant deviation from the control (Table II).

Although myocardial glucose extraction and usage during normovolemic shock showed statistically no significant deviation from the control, it is apparent from Table II and Figure 2 that in a number of experiments, the glucose concentration of coronary vein blood still exceeded that of arterial blood (13–16, 24 and Table II).

In three experiments in which metabolic changes were observed at frequent (30-minute)

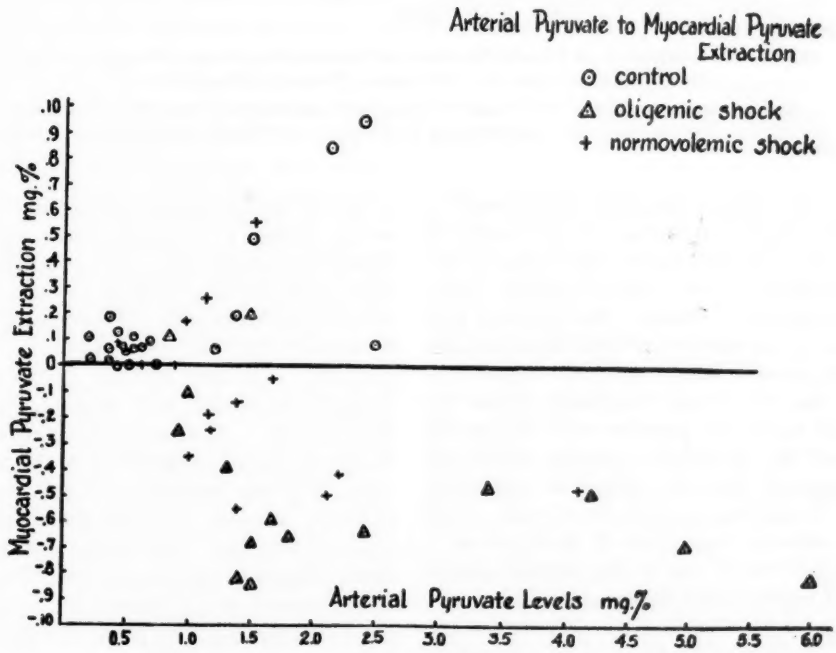

FIG. 4. THE RELATIONSHIP OF ARTERIAL CONCENTRATIONS OF PYRUVATE TO MYOCARDIAL PYRUVATE EXTRACTION

The arterial concentrations of pyruvate are elevated during both oligemic and normovolemic shock. Many of the points fall below the zero line, illustrating that pyruvate concentration in coronary vein blood exceed those in coronary arterial blood in many instances. This "pyruvate reversal" is present during both phases of shock.

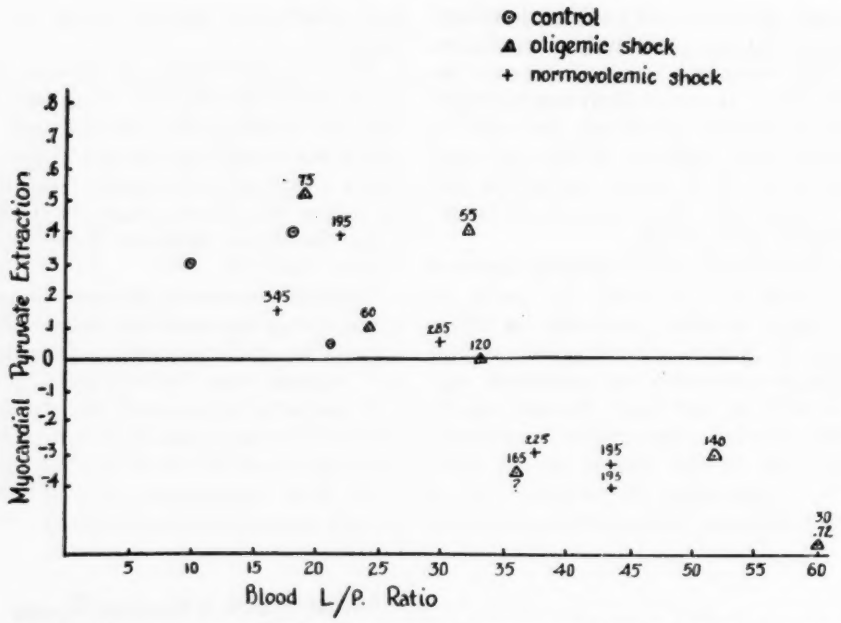

FIG. 5. ILLUSTRATION OF AN INVERSE RELATIONSHIP BETWEEN THE RATIO OF ARTERIAL LACTATE TO PYRUVATE AND THE MYOCARDIAL PYRUVATE EXTRACTION

An increase in the ratio of blood lactate to pyruvate is accompanied by a negative pyruvate extraction (coronary pyruvate concentrations in coronary vein blood exceed those in arterial blood).

intervals, the findings described above could be confirmed. Table III illustrates a rise in arterial glucose, pyruvate and lactate concentration during the oligemic phase, which is partially maintained during normovolemia. The pyruvate concentration in coronary vein blood exceeded that of arterial blood in many instances. Figure 5 illustrates that an inverse relationship existed between the lactate to pyruvate ratio in arterial blood and the myocardial pyruvate extraction. This suggested that the change in myocardial pyruvate metabolism was associated with a shift toward anaerobic metabolism of the organism.

There was also a rise in the arterial concentration of ketone bodies during oligemic and normovolemic shock (Table III). Myocardial extraction of ketones, however, was not greatly altered (Table III).

DISCUSSION

Results on the dynamic changes of hemorrhagic shock confirm the results of previous investigators. A fall in cardiac output and stroke volume observed during the oligemic and normovolemic phase has been noted in experimental hemorrhagic shock by Wiggers and Werle (3) and in clinical shock by Richards (37). The resulting "stagnant anoxemia" also has been repeatedly described (29, 30). The data reported in this paper are in partial agreement with those of Opdyke and Foreman (6). Their experiments indicated that during the period of hypotension, the actual coronary flow was seriously curtailed; immediately following infusion, however, the coronary flow appeared adequate. The results reported in this report illustrate that the coronary flow remains below control levels during the normovolemic phase contributing to the persistence of myocardial ischemia.

It must be stressed that the degree of myocardial anoxia is less severe than that encountered in ventricular fibrillation where the cardiac output is below 10 per cent of normal (38). It is likely that the metabolic response of the myo-

cardium varies with the degree of ischemia. In complete myocardial ischemia, glycolysis occurs and lactate is produced by the muscle. Glucose and potassium levels of coronary vein blood are also higher than those in arterial blood (39). Furthermore, in complete anoxia, the coronary arteriovenous oxygen difference is significantly reduced (39). A reduction in oxygen consumption of the heart is not synonymous with myocardial anoxia. However, it is likely that the metabolic changes in the myocardium can result from myocardial ischemia alone. An important factor in determining the metabolic response of the myocardium in the presence of reduced coronary flow is the ratio of oxygen demand of the heart muscle to its oxygen supply. If oxygen demand and supply are proportionally diminished, the metabolic effect of myocardial ischemia is slight. Within the conditions of these experiments, the metabolic requirements of the myocardium are probably not reduced; furthermore, although under normal conditions the energy liberation of the heart appears to vary directly with energy utilization, this relationship is broken in certain pathologic conditions such as myocardial failure (32). It is unlikely that in the hearts of animals suffering from hemorrhagic shock, supply and demand for oxygen are in equilibrium. This is in contrast to the effect of a reduction in body temperature (hypothermia). There, metabolic disturbances do not develop because the reduction in body temperature lessens oxygen demands and supply in the same proportion (31).

Myocardial ischemia is, therefore, present throughout the various phases of shock and might well show its result in irreversible myocardial failure. Similar conclusions have been reached by Wiggers (1) and by Case and Sarnoff (40). The latter workers showed that left atrial pressure fell to normal if the left descending coronary artery was perfused at normal arterial pressure in the presence of low systemic arterial pressure (40). Characteristic deviations of S-T segment and T wave in the electrocardiogram during oligemic shock and irreversible normovolemic shock are further support of the presence of myocardial ischemia (41).

In contrast, myocardial oxygen extraction showed no significant change during the oligemic and normovolemic phase of shock (Table I, Figure 1). It had been shown in previous studies that the changes in coronary blood flow and not the myocardial oxygen extraction are responsible for alterations in myocardial oxygen consumption (31, 32). In most organs, a reduction of blood flow is at least partially compensated for by increased oxygen extraction. This appears not to be the case for the heart.

The coronary vascular resistance falls during the oligemic phase and increases during normovolemia (Table I). The mechanism of increased coronary vascular resistance during normovolemia is difficult to understand. Such a decrease in coronary vascular resistance would not seem fortuitous in replenishing the ischemic myocardium. In contrast, Opdyke and Foreman, using a coronary artery perfusion set in open chest dogs, observed a marked decrease in coronary resistance following reinfusion (6).

Since left ventricular work falls proportionally more than left ventricular oxygen consumption, the cardiac efficiency declines during oligemic shock (Table I). Retransfusion does not result in any uniform change in myocardial efficiency (Table I). It is likely that the persistence of low myocardial efficiency in some of the animals indicates the presence of myocardial failure. The observation that not all hearts show this decline in their mechanical efficiency is in line with the findings of Wiggers who detected evidence of myocardial failure as demonstrated by rising effective filling pressure in a majority but not all dogs in normovolemic failure (1).

Metabolic studies demonstrate a significant rise in the arterial concentration of glucose, lactate, and pyruvate during oligemic shock (Table II, Figures 2–4). During the normovolemic phase the blood concentration of glucose falls, while those of pyruvate and lactate remain elevated (Table II, Figures 2–4). These findings are in line with those of other investigators (9–12). It is likely that hyperglycemia is the result of glycogenolysis with depletion of liver glycogen (1). Glycogenolysis is also accelerated by the acidosis. The role played by epinephrine in this mechanism is not yet clear (42). The rise in arterial lactate concentration is probably the result of a shift toward anaerobic metabolism (1). Acceleration of lactate production by the muscle and gradual failure of the liver to remove lactate may

play a role in elevating blood lactate. If the heart responded to anoxia as other organs by an increased production of lactate *via* the usual glycolytic scheme, one might expect little lactate utilization and even an increased concentration of lactate in the coronary venous blood as compared to the arterial blood. From results obtained in this paper, it would seem that myocardial extraction of lactate is greatly increased during both oligemic and normovolemic shock, although its myocardial usage is not altered because of the fall in coronary blood flow. Lactate is utilized by the isolated heart and by the human and dog's heart *in situ* (31, 33, 43). Increased extraction by the heart in shock indicates that the enzymatic pathways through which lactate is channeled are not interfered with by the myocardial ischemia in hemorrhagic shock. On the basis of results discussed below, an increased breakdown of lactate through the tricarboxylic acid cycle seems unlikely, because decarboxylation of pyruvate is blocked. A conversion of lactate through reversal of the glycolytic reaction remains, therefore, a possibility although Greig had demonstrated disappearance of cozymase following hemorrhagic shock (15).

The other possibility is that lactate is oxidized during enzymatic action of lactic acid oxidase I which has recently been isolated from mycobacterium tuberculosis avium (44). Only molecular oxygen is required for the action of this enzyme and addition of cytochrome c, cytochrome oxidase is not necessary. Furthermore, the enzyme is not dependent upon the addition of A.T.P., D.P.N., or cocarboxylase.

The trend toward a negative myocardial balance of pyruvate during both phases of shock could conceivably result from destruction of the coenzyme cocarboxylase. Ochoa first observed that in experiments with liver, kidney, muscle and brain, cocarboxylase is destroyed under anaerobic conditions, by an enzyme, probably a phosphatase (14). Greig and Govier demonstrated in dogs in hemorrhagic shock that the cocarboxylase content of muscle, liver and duodenum decreased (13). The disappearance of the coenzyme was thought to result from its dephosphorylation, which occurred most frequently in muscle. Similar results were obtained, when the animals were made anoxic by inhalation of 10 per cent oxygen mixtures (13). It thus appears that anaerobiasis, as a common factor in both shock and anoxic anoxia, is responsible for dephosphorylation of cocarboxylase. Although direct determinations of cocarboxylase in heart muscle have not been made in this investigation, the findings of negative myocardial pyruvate balance are consistent with a destruction of this coenzyme in heart muscle. This destruction, resulting from increased dephosphorylation of the coenzyme, is possibly the result of a diminution in coronary blood flow. The importance of anaerobiasis in the production of this metabolic disturbance is illustrated in Figure 5 in which it may be seen that an inverse relationship exists

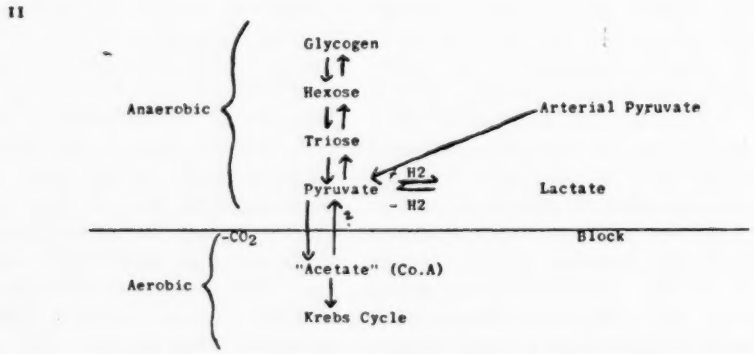

FIG. 6. METABOLIC SCHEME ILLUSTRATING THE METABOLIC BLOCK WHICH RESULTS FROM INHIBITION OF COCARBOXYLASE

The metabolic break occurs between pyruvate and "acetate," thus eliminating the aerobic Krebs cycle.

between the lactate to pyruvate ratio and the myocardial pyruvate extraction.

Changes in glucose metabolism are more difficult to interpret. The most conspicuous finding is that of reduced myocardial extraction and usage of glucose in the presence of increased arterial glucose concentration (Table II, Figure 2). In several instances there is also a negative myocardial glucose balance. These findings might be interpreted as suggesting either diminished myocardial glucose utilization, or gluconeogenesis by the heart muscle with glucose escaping into the coronary venous blood. Any conclusions are at the moment not possible.

The results reported in this paper illustrate the metabolic response of the intact heart to hemorrhagic shock and in a more specific sense to myocardial ischemia. The data suggest that moderate ischemia, resulting from a diminution in coronary blood flow, can bring about specific metabolic changes in the myocardium. Figure 6 illustrates that, assuming partial or complete inhibition of cocarboxylase, the metabolic break occurs between pyruvate and "acetate." This does not mean that other aerobic metabolic processes not connected with the action of cocarboxylase are interfered with. Thus, oxidation of fatty acids and the attachment of fatty acids to coenzyme A may proceed in a normal fashion, furnishing the high energy phosphate needed in the glycolytic cycle. However, this possibility is unlikely because the flavin cofactor (F.A.D.) required for the first step in fatty acid catabolism cannot be reoxidized under anaerobic conditions as is the case for the pyridine cofactor (D.P.N.) involved in glycolysis.

SUMMARY

Cardiac output, stroke volume and coronary blood flow were diminished during oligemic and normovolemic phase of hemorrhagic shock.

As a result of diminished coronary flow, the myocardial oxygen usage was significantly reduced during both phases of shock.

The myocardial oxygen extraction showed no significant change during the oligemic and normovolemic phase of shock.

The coronary vascular resistance fell during the oligemic phase and increased during normovolemia.

Cardiac efficiency declined during oligemia; there was no uniform change in cardiac efficiency after retransfusion of blood.

Arterial concentrations of pyruvate and lactate were elevated during oligemic and normovolemic shock. The arterial glucose concentration which had risen during oligemia, declined during normovolemia.

The myocardial extraction of lactate was increased during hemorrhagic shock. In contrast, elevation of arterial blood glucose concentration did not result in an increase in myocardial glucose extraction. In some instances, glucose concentration in coronary venous blood exceeded that in arterial blood.

Myocardial pyruvate extraction was diminished, and frequently the pyruvate concentration in coronary vein blood exceeded that in arterial blood.

These data suggest that myocardial ischemia, resulting from a diminution in coronary flow can bring about specific metabolic changes in the heart muscle, as for example, destruction of cocarboxylase.

ACKNOWLEDGMENT

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REFERENCES

1. Wiggers, C. J., Physiology of Shock. New York, The Commonwealth Fund, 1950, 459 p.
2. Wiggers, C. J., The failure of transfusions in irreversible hemorrhagic shock. (A study of central venous pressures). Am. J. Physiol., 1945, **144**, 91.
3. Wiggers, C. J., and Werle, J. M., Cardiac and peripheral resistance factors as determinants of circulatory failure in hemorrhagic shock. Am. J. Physiol., 1942, **136**, 421.
4. Kohlstaedt, K. G., and Page, I. H., Terminal hemorrhagic shock; circulatory dynamics, recognition, and treatment. Surgery, 1944, **16**, 430.
5. Blalock, A., Principles of Surgical Care, Shock and Other Problems. St. Louis, Mosby Company, 1940, 325 p.
6. Opdyke, D. F., and Foreman, R. C., A study of coronary flow under conditions of hemorrhagic hypotension and shock. Am. J. Physiol., 1947, **148**, 726.
7. Seligman, A. M., Frank, H. A., Alexander, B., and Fine, J., Traumatic shock. XV. Carbohydrate metabolism in hemorrhagic shock in the dog. J. Clin. Invest., 1947, **26**, 536.

8. Hamilton, J. I., and Haist, R. E., Studies on experimental shock in dogs. Canad. J. Research, Sect. E, 1945, **23**, 89.
9. Riegel, C., Formation of lactic acid in the body after severe hemorrhage. J. Biol. Chem., 1927, **74**, 123.
10. Swan, M. M., The effect of severe hemorrhage, seasonal temperature and diurnal variation on blood lactate in the dog. Am. J. Physiol., 1943, **140**, 125.
11. McShan, W. H., Potter, V. R., Goldman, A., Shipley, E. G., and Meyer, R. K., Biological energy transformations during shock as shown by blood chemistry. Am. J. Physiol., 1945, **145**, 93.
12. Aub, J. C., and Wu, H., Studies in experimental traumatic shock. III. Chemical changes in the blood. Am. J. Physiol., 1920, **54**, 416.
13. Greig, M. E., and Govier, W. M., Studies on shock induced by hemorrhage. VI. Cocarboxylase phosphatase in dog serum. J. Pharmacol. & Exper. Therap., 1943, **79**, 246.
14. Ochoa, S., Enzymic synthesis of cocarboxylase in animal tissues. Biochem. J., 1939, **33**, 1262.
15. Greig, M. E., Studies on shock induced by hemorrhage. VII. The destruction of cozymase and alloxazine adenine dinucleotide in tissues during shock. J. Pharmacol. & Exper. Therap., 1944, **81**, 164.
16. De Turk, W. E., and Greig, M. E., Studies on shock induced by hemorrhage. IX. The inhibition of amino acid oxidation in shock induced by hemorrhage. J. Pharmacol. & Exper. Therap., 1945, **83**, 220.
17. Bing, R. J., Hammond, M. M., Handelsman, J. C., Powers, S. R., Spencer, F. C., Eckenhoff, J. E., Goodale, W. T., Hafkenschiel, J. H., and Kety, S. S., The measurement of coronary blood flow, oxygen consumption, and efficiency of the left ventricle in man. Am. Heart J., 1949, **38**, 1.
18. Goodale, W. T., and Hackel, D. B., Measurement of coronary blood flow in dogs and man from rate of myocardial nitrous oxide desaturation. Circulation Research, 1953, **1**, 502.
19. Van Slyke, D. D., and Neill, J. M., The determination of gases in blood and other solutions by vacuum extraction and by manometric measurement. J. Biol. Chem., 1924, **61**, 523.
20. Haldane, J. S., Methods of Air Analysis. London, Charles Griffin and Co., Ltd., 1912, 130 p.
21. Hagedorn, H. C., and Jensen, B. N., Zur Mikrobestimmung des Blutzuckers mittels Ferricyanid. Biochem. Ztschr., 1923, **135**, 46.
22. Somogyi, M., A method for the preparation of blood filtrates for the determination of sugar. J. Biol. Chem., 1930, **86**, 655.
23. Friedemann, T. E., and Haugen, G. E., Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem., 1943, **147**, 415.
24. Barker, S. B., and Summerson, W. H., The colorimetric determination of lactic acid in biological material. J. Biol. Chem., 1941, **138**, 535.
25. Greenberg, L. A., and Lester, D., A micromethod for the determination of acetone and ketone bodies. J. Biol. Chem., 1944, **154**, 177.
26. Spencer, F. C., Merrill, D. L., Powers, S. R., and Bing, R. J., Coronary blood flow and cardiac oxygen consumption in unanesthetized dogs. Am. J. Physiol., 1950, **160**, 149.
27. Herrmann, G. R., Experimental heart disease. I. Methods of dividing hearts; with sectional and proportional weights and ratios for two hundred normal dogs' hearts. Am. Heart J., 1925, **1**, 213.
28. Fisher, R. A., Statistical Methods for Research Workers, 8th ed., Edinburgh, Oliver and Boyd, 1941, 344 p.
29. Price, P. B., Hanlon, C. R., Longmire, W. P., and Metcalf, W., Experimental shock. I. Effects of acute hemorrhage in healthy dogs. Bull. Johns Hopkins Hosp., 1941, **69**, 327.
30. Beecher, H. K., McCarrell, J. D., and Evans, E. I., A study of the "shock-delaying" action of the barbiturates with a consideration of the failure of oxygen-rich atmospheres to delay the onset of experimental shock during anesthesia. Ann. Surg., 1942, **116**, 658.
31. Edwards, W. S., Tuluy, S., Reber, W. E., Siegel, A., and Bing, R. J., Coronary blood flow and myocardial metabolism in hypothermia. Ann. Surg., 1954, **139**, 275.
32. Bing, R. J., and Daley, R., Behavior of the myocardium in health and disease as studied by coronary sinus catheterization. Am. J. Med., 1951, **10**, 711.
33. Bing, R. J., Siegel, A., Vitale, A., Balboni, F., Sparks, E., Taeschler, M., Klapper, M., and Edwards, S., Metabolic studies on the human heart in vivo. I. Studies on carbohydrate metabolism of the human heart. Am. J. Med., 1953, **15**, 284.
34. Bing, R. J., Siegel, A., Ungar, I., and Gilbert, M., Metabolism of the human heart. II. Studies on fat, ketone and amino acid metabolism. Am. J. Med., 1954, **16**, 504.
35. Govier, W. M., and Greer, C. M., Studies on shock induced by hemorrhage. II. Effect of thiamin on disturbances of carbohydrate metabolism. J. Pharmacol. & Exper. Therap., 1941, **72**, 321.
36. LePage, G. A., The effects of hemorrhage on tissue metabolites. Am. J. Physiol., 1946, **147**, 446.
37. Richards, D. W., Jr., The circulation in traumatic shock in man. Bull. New York Acad. Med., 1944, **20**, 363.
38. Southworth, J. L., Pierce, E. C., II, Rawson, F. L., Jr., and McKusick, V. A., The state of the circulation during ventricular fibrillation. Surgical Forum, Proceedings of the 36th Clinical Congress of the American College of Surgeons, 1950, p. 221.

39. Rich, A., Vaughn, B. J., and Bing, R. J., Myocardial metabolism in ventricular fibrillation. In preparation.
40. Case, R. B., and Sarnoff, S. J., Insufficient coronary flow and consequent myocardial failure as a contributing factor in hemorrhagic shock. Federation Proc., 1953, (Abstract no. 72), **12**, 24.
41. Master, A. M., Dack, S., Grishman, A., Field, L. E., and Norn, H., Acute coronary insufficiency: an entity; shock, hemorrhage and pulmonary embolism as factors in its production. J. Mt. Sinai Hosp., 1947, **14**, 8.
42. Engel, F. L., Winton, M. G., and Long, C. N. H., Biochemical studies on shock. I. The metabolism of amino acids and carbohydrate during hemorrhagic shock in the rat. J. Exper. Med., 1943, **77**, 397.
43. Evans, C. L., The metabolism of cardiac muscle *in* Evans, C. L., ed., Recent Advances in Physiology, rev. by W. H. Newton, 6th ed., Philadelphia, Blakiston Company, 1939, p. 157–215.
44. Yamamura, Y., Kusunose, M., and Kusunose, E., Lactic oxidases of *mycobacterium tuberculosis avium*. Nature, 1952, **170**, 207.

PROTEIN-LIPID RELATIONSHIPS IN HUMAN PLASMA. III. IN PREGNANCY AND THE NEWBORN [1]

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This study of pregnant women and their newborn infants was undertaken as a part of a more extensive investigation of factors that modify the interrelations of proteins and lipids in human plasma (1–3).

The composition of maternal and fetal blood has been examined by many observers who, however, have usually focussed attention either on proteins or lipids. Electrophoretic studies of proteins have been made by Longsworth, Curtis, and Pembroke (4) who analyzed both serum and plasma; by Lagercrantz (5) and Moore, DuPan, and Buxton (6) who examined only serum; and by Macy and Mack (7) whose monograph was devoted to an inquiry concerning plasma proteins in human reproduction. There is consensus that maternal plasma has less than normal concentration of albumin and a greater than normal concentration of alpha and beta globulins while gamma globulins are unchanged or only slightly decreased. These electrophoretic analyses of the cord plasma have shown lower than normal concentration of total protein which is chiefly attributable to a marked reduction in the concentration of alpha and beta globulins. Numerous investigators (8–10) have shown that in maternal blood the concentration of cholesterol and phospholipids is greater than normal while in blood from the umbilical cord at the time of birth it is notably reduced (11–13).

In the present study, utilization of the microfractionation method No. 10 of Cohn and his coworkers (14) has made possible the separation of the lipoproteins of plasma into two fractions which together contain essentially all of the lipids. By such separation and by subsequent analyses of the protein and lipid constituents of each fraction, it is possible to define their relationships in a manner not attainable by analyses of the unfractionated plasma.

SUBJECTS

Twenty-seven pregnant women who were free from detectable abnormalities during their prepartum examination at The New York Hospital served as subjects for the study. They varied in age from 19 to 40; 13 were primipara and 4 were completing their fourth pregnancy. From each subject blood samples were taken immediately following the delivery. In 25 of the cases observations were made on blood samples drawn simultaneously from the placental end of the umbilical cord.

METHODS

The microfractionation method of Cohn and his coworkers (14) was used for separation of proteins. In this study the procedure was carried out in only three stages with isolation of Fraction IV + V + VI, Fraction II, and Fraction I + III. Analyses for protein, cholesterol, and phospholipids were made on the unfractionated plasma and each of its fractions. Because of the extremely small amounts of phospholipids in Fraction II, the determination was omitted.

Details of the procedure involving fractionation and the analytical methods for proteins, cholesterol, and phospholipids have been presented in a previous publication (1). They have not been significantly modified for the present work.

The distribution of protein components in the various fractions was studied electrophoretically by the moving boundary method and also by paper ionophoresis which was adapted to the purpose of identifying lipoproteins.

The moving boundary method was performed on ACD plasma and the three fractions using an Aminco Stern apparatus with a standard analytical cell. Details of this procedure and the preparation of samples prior to electrophoresis have been described in a previous publication (1).

Paper electrophoresis was carried out by a modification and combination of several methods (15–17). A vapor tight plastic chamber was employed and the experiments were conducted at 20 to 25° C. using veronal-citrate buffer pH 8.6, $\Gamma/2$ 0.05; a rectangular sheet of Whatman 3

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TABLE I

Concentration and distribution of proteins, cholesterol, and phospholipids in (A) maternal plasma, (B) cord plasma

Subject	Age and Sex	Total protein *Gm./100 ml.* Kjeldahl	Protein in fractions *Gm./100 ml.* Biuret IV+V+VI	II	I+III	Total cholesterol *Mg./100 ml.*	Cholesterol in fractions *Mg./100 ml.* IV+V+VI	II	I+III	Total phospholipid *Mg./100 ml.*	Phospholipid in fractions *Mg./100 ml.* IV+V+VI	I+III
A. Maternal Plasma at Time of Delivery												
Lee	20	6.20	3.97	0.56	1.86	250	64	0	177	305	131	146
Hof	22	7.40	4.30	0.70	2.58	439	68	3	358		129	279
Wal	21	6.20	3.88	0.63	1.89	291	51	2	216	387	116	237
Arb	30	6.87	4.50	0.68	2.20	280	60	4	195	357	142	192
Fuh	27	6.56	4.25	0.65	2.20	300	77	1	205	405	187	202
Wil	27	6.20	3.74	0.69	2.22	231	28	2	184	278	82	183
Cly	27	7.02	4.43	0.91	2.00	356	83	4	262			
Coh	21	6.61	4.28	0.88	1.96	250	57	1	181			
Vog	20	6.40	3.86	0.71	2.09	236	41	5	189	330	107	207
Lub	24	6.70	4.12	0.67	2.08	281	77	4	200			
Lau	24	6.68	4.12	0.78	1.94	270	75	5	183			
Wer	39	7.13	4.33	0.47	2.57	485	48	4	402	600	118	370
Lea	39	6.74	4.42	0.77	1.82	244	78	0	152			
Nor	23	5.08	3.08	0.44	1.76	209	27	3	167			
Lug	26	6.41	4.31	0.80	1.89	292	64	2	219	373	118	247
Sch	35	6.82	4.51	0.59	2.03	283	78	2	192	390	178	167
Har	40	7.18	3.98	1.13	2.34	283	71	10	197	346	127	171
Bro	20	6.48	4.45	0.95	2.05	314	71	5	229	316	117	171
Gil	30	6.36	4.06	0.77	1.90	222	88	3	125	331	177	119
Sev	33	5.99	3.82	0.64	1.69	254	53	3	196			
San	21	6.87	4.42	0.59	2.19	321	57	1	263			
Sta	22	6.31	4.10	0.60	2.13	247	45	0	203			
Mot		6.60	4.26	0.67	1.81	252	83	0	143			
Mah	22	6.13	4.24	0.61	1.58	235	46	0	191			
Hef	24	6.96	4.24	0.62	2.31	296	106	0	194			
Pop	22	7.27	4.66	0.70	2.24	274	64	4	210			
Tor	21	6.20	4.18	0.54	2.05	226	55	4	164			
Mean		6.57	4.17	0.69	2.05	282	63	3	207	372	139	206
S.D.		±0.51	±0.32	±0.15	±0.24	±62.2	±18.2	±2.3	±58.5	± 80	± 34	± 63
Normal non-pregnant women (aged 18–35)												
Mean		6.71	4.83	0.81	1.34	189	61	3	123	225	117	98
S.D.		±0.27	±0.25	±0.14	±0.13	± 35	±13		± 32	± 28	± 20	± 23
P		<0.3	<0.01	<0.01	<0.01	<0.01	<0.6		<0.01	<0.01	<0.02	<0.01
B. Cord Plasma												
Lee	M	6.50	4.36	1.18	1.34	85	27	1	38	130	68	51
Hof	F	5.70	4.16	0.83	0.91	67	26	0	41	105	50	40
Wal	F	5.60	3.98	1.05	0.81	53	14	0	33	122	51	45
Arb	F	6.38	4.42	1.09	1.25	85	31	4	42	138	69	53
Fuh	F	5.46	3.93	0.79	0.97	78	28	2	40	134	65	46
Wil	F	5.81	4.32	0.81	0.96	81	27	0	44	145	83	45
Cly	F	5.56	4.07	0.87	0.83	48	23	0	26			
Coh	F	6.37	4.49	1.16	0.94	73	33	0	38			
Vog	F	5.70	3.95	1.14	0.79	48	28	3	15	93	68	9
Lut	M	6.44	4.43	1.21	1.08	81	38	3	38			
Lau	M	5.42	3.94	0.78	0.97	60	24	4	34			
Wer	M	5.02	3.69	0.55	0.82	48	25	2	20	105	62	35
Lea	F	6.24	4.26	1.15	1.07	98	26	0	65			
Nor	F	4.64	3.21	0.64	1.04	71	32	0	38	112	63	30
Lug	F	5.43	4.23	0.91	0.85	64	35	0	29	162	70	43
Sch		5.79	4.26	0.81	1.06	82	27	2	53	151	84	27
Har	M	5.78	4.13	1.02	0.92	76	48	3	28	103	58	42
Bro	M	6.07	4.12	1.13	1.09	67	27	3	37	106	65	37
Gil	M	6.07	4.22	1.01	0.98	65	29	4	31			
Sev		5.21	3.92	0.81	0.84	50	16	0	27			
San		5.55	3.98	0.79	1.07	58	29	0	29			
Sta	M	5.71	4.00	1.01	1.08	52	29	0	23			
Mot	M	5.61	3.26	0.81	0.74	47	14	0	29			
Mah		6.10	4.50	0.89	0.83	89	40	0	41			
Hef	F	4.93	3.72	0.64	1.11	79	30	0	51			
Mean		5.72	4.06	0.92	0.97	68	28	1	36	124	66	39
S.D.		±0.48	±0.33	±0.19	±0.15	±15	± 8	±1	±11	± 22	±10	±12

MM filter paper (17 × 35 cm.) was suspended in a taut, horizontal position across two plastic rods spaced 18 cm. apart in a movable plastic frame. The buffer vessels (4 × 4 × 18 cm.) were connected with KCl-agar salt bridges to 100 ml. beakers containing platinum electrodes.

Prior to electrophoresis, the fractions were concentrated to approximately 4.0 gm. per 100 ml. protein solution in the same manner as described for the moving boundary method (1) but dialysis was not essential.

One-hundredth of a milliliter of ACD plasma and each of the concentrated fractions (IV + V + VI and I + III) were applied 1.5 cm. apart in duplicate groups, thus making a total of eight samples per sheet of filter paper. A potential of approximately 7 to 9 volts per cm. was supplied by a variable-voltage vacuum tube rectifier operating from a 110 AC line.

Under these conditions complete resolution of the protein components was obtained after approximately five and one-half hours. The paper was dried in an oven and cut into two sections. One section (plasma, Fractions IV + V + VI, II, and I + III) was stained with bromphenol blue for all protein components according to Goa (18); the duplicate section was stained with Sudan black B for lipoproteins according to Swahn (19).

RESULTS

Data of the fractionation and analyses of maternal plasma are presented in Table IA which also contains for sake of comparison mean values with standard deviation of 21 normal non-pregnant young women. Results on the cord plasma are recorded separately in Table IB but may be identified with those of the respective mothers by the symbols in the first column of the table.

Maternal Plasma

Protein concentration and distribution

There is little difference in the mean values for total protein in the normal and pregnant group, although the range among the pregnant women is somewhat greater. Variations in the concentration in Fraction IV + V + VI are considerable but the values are with one exception below the normal range. The concentration of proteins in Fraction II is significantly less than in the normal group, and in Fraction I + III it greatly exceeds that of non-pregnant women.

Concentration and distribution of cholesterol and phospholipid

In the plasma of pregnant women the concentrations of both cholesterol and phospholipid are greater than normal. The increment in cholesterol occurs entirely in the cholesterol in Fraction I + III. The cholesterol in Fraction IV + V + VI is virtually identical in both groups. Also, with the phospholipids almost all the increase in the plasma concentration is found in Fraction I + III. In Table II are shown the derived data for the

TABLE II

Distribution of cholesterol and phospholipids and cholesterol-phospholipid ratios in maternal and cord plasma and fractions compared with similar values in the plasma of young non-pregnant women

	Plasma Cholesterol-phospholipid ratio	Fraction IV+V+VI			Fraction I+III		
		per cent of total cholesterol	*per cent* of total phospholipids	Cholesterol-phospholipid ratio	*per cent* of total cholesterol	*per cent* of total phospholipids	Cholesterol-phospholipid ratio
Pregnant women 14 Subjects Aged 20–40							
Mean	0.84	23.7	40.8	0.47	75.5	59.2	1.07
S.D.	±0.11	±7.3	±10.2	±0.08	±8.3	±9.9	±0.14
Range	0.73–1.06	10.2–40.9	24.2–59.9	0.34–0.61	57.7–88.6	40.1–75.8	0.89–1.34
Non-pregnant women 24 Subjects Aged 19–35							
Mean	0.84	33.2	54.5	0.52	65.2	45.2	1.25
S.D.	±0.11	±8.9	±7.2	±0.06	±8.9	±8.0	±0.16
Range	0.59–1.08	22.0–61.0	38.2–78.3	0.40–0.64	37.0–77.9	22.0–56.7	0.91–1.56
12 Cord plasmas							
Mean	0.57	43.3	63.1	0.44	55.0	36.9	0.87
S.D.	±0.08	±8.7	±9.5	±0.08	±9.6	±9.5	±0.14
Range	0.44–0.66	28.7–61.0	55.1–88.5	0.28–0.57	33.0–71.3	11.5–44.9	0.57–1.07

maternal plasma. The percentage of total cholesterol in Fraction IV + V + VI is highly variable with many values within the normal range but with few considerably below it. The mean of 23.7 per cent is considerably below the normal mean of 33.2 per cent. Conversely, the mean percentage of cholesterol in Fraction I + III is increased above normal in the pregnant women.

The mean cholesterol-phospholipid ratio in whole plasma does not differ in the two groups. The ratio in Fraction I + III is somewhat variable but the mean is significantly lower than normal. This does not depress the plasma ratio because of the greater proportion of cholesterol and phospholipid in Fraction I + III.

Cord Plasma

Protein concentration and distribution

In the newborn infant the concentration of total protein is definitely less than that of the mother or of normal non-pregnant young women. In Fraction IV + V + VI, also, the concentrations of protein are slightly lower than the adult values although the percentage relative to total protein tends to be higher and approximates that of normal young adults. In Fraction II in which gamma globulins are concentrated, the values exceed considerably those of the mothers and of healthy non-pregnant young women. Both the concentration of protein and the percentage of the total protein in Fraction I + III average about double those of normal.

Concentration and distribution of cholesterol and phospholipid

In cord plasma, cholesterol and phospholipid concentrations are very much lower than those found in adults. The absolute amount of cholesterol in Fraction IV + V + VI is about half that in normal young women; the cholesterol in Fraction I + III is about one-third that found in the normal females.

Cholesterol was usually recovered well in the fractions. The recovery of phospholipid was less complete. This may have been due in part to the omission of analysis in Fraction II. Where the total phospholipid is so low, loss of even a small amount is proportionately large. Because of the low concentrations of both cholesterol and phospholipids relatively small errors in analysis were extremely consequential in the calculation of cholesterol-phospholipid ratios.

In Table II, the means of the relative concentrations of cholesterol and phospholipid in the two fractions are shown together with the mean values for the cholesterol-phospholipid ratios. It will be seen that there is a high relative concentration of cholesterol in Fraction IV + V + VI and a corresponding diminution in Fraction I + III. The cholesterol-phospholipid ratios are within the normal range in Fraction IV + V + VI but considerably reduced in whole plasma and in Fraction I + III.

Electrophoresis

Moving boundary analysis of the plasma and fractions from normal pregnant women revealed the various electrophoretic components which are indicated in Table III as percentage area distribution. For comparison, the values of a normal young woman are included in the table. It may be seen that in maternal plasma the protein separation is of the same type as that obtained by Lever and his co-workers (20) and ourselves (1) for normal individuals. The albumin is concentrated chiefly in Fraction IV + V + VI with occasional trace amounts in Fractions II and I + III; gamma globulin is found mainly in Fraction II with slightly greater than normal loss into Fraction I + III; all of the fibrinogen is localized in Fraction I + III where also variable amounts of alpha and beta globulin appear.

The observations from paper electrophoresis with Sudan black B staining show that in both the normal non-pregnant and pregnant women all of the lipids in Fraction IV + V + VI are concentrated in the $alpha_1$-albumin area, while those in Fraction I + III of the pregnant women appear in a slightly faster than normal $beta_1$ globulin position. Lipids are demonstrable only occasionally in Fraction II where they are barely perceptible throughout the $beta_1$ gamma globulin areas.

It is evident from the table that the percentage of albumin is lower, and that of $alpha_1$, $alpha_2$, and $beta_1$ globulins is higher in normal pregnant women. The decrease in albumin is reflected in

TABLE III

Moving boundary electrophoretic analysis of plasma and fractions from pregnant and non-pregnant women. A comparison of percentage area distribution of protein components

Specimen	Subject Women 19–40 yrs.	Albumin (%)	α_1 (%)	α_2 (%)	β_1 (%)	β_2 (%)	ϕ (%)	γ (%)
Plasma	Normal non-pregnant	51.5	10.9	8.8	11.4	—	7.2 —	10.1
	Normal pregnant	42.9	11.5	10.0	17.8	—	8.3 —	9.6
	Normal pregnant	33.5	13.3	13.4	18.1	—	8.9 —	12.9
	Normal pregnant	40.5	14.9	11.8	16.5	—	8.1 —	8.2
Fraction IV+V+VI	Normal non-pregnant	81.9	9.8	4.6	3.6	—	—	—
	Normal pregnant	72.9	14.5	6.1	6.6	—	—	—
	Normal pregnant	68.5	17.0	6.7	7.9	—	—	—
	Normal pregnant	65.5	17.3	9.6	7.6	—	—	—
Fraction II	Normal non-pregnant	—	—	—	—	12.7	—	87.4
	Normal pregnant	3.2	—	—	5.5	11.0	—	80.4
	Normal pregnant	3.0	—	—	—	12.6	—	84.3
	Normal pregnant	—	—	—	—	—	—	—
Fraction I+III	Normal non-pregnant	—	4.6	25.6	25.1	12.8	23.2	8.7
	Normal pregnant	—	10.4	43.5	5.9	10.9	20.9	9.0
	Normal pregnant	7.6	17.9	29.7	10.4	9.9	15.1	9.4
	Normal pregnant	—	8.9	48.9	13.9	—	17.2 —	10.8

Fraction IV + V + VI where there is also a moderate increase in alpha$_1$, alpha$_2$, and beta$_1$ globulins.

It should be mentioned that our values for percentage of total area as alpha$_1$ globulin in plasma and in Fraction IV + V + VI of normal individuals recorded here and in a previous paper (1) are somewhat higher than those reported by other observers (7, 20). This may be attributed to the difficulties encountered in precise measurement of the alpha$_1$ area which frequently resolves poorly in veronal-citrate buffer.

In Fraction II the percentage of gamma globulin is slightly lower than normal. In Fraction I + III variable amounts of the plasma increment of non-lipid-bearing alpha$_1$ globulin appear. The increased percentage of alpha$_2$ globulin in this fraction is striking particularly in relation to the relative values for beta$_1$ globulin which are not ostensibly augmented. The disproportion is perhaps more apparent than real and may be due in part to the faster than normal mobility of the beta$_1$ components. The migration of the beta$_1$ peak as a long thin shoulder of a large alpha$_2$ peak under which it is more than particularly buried, makes calculation of the two areas extremely difficult. All of the lipids in Fraction I + III as determined by paper electrophoresis are concentrated in this alpha$_2$–beta$_1$ area. The fibrinogen recovered in Fraction I + III is variable in amount but unremarkable.

Cord plasma and its fractions have been analyzed only with the method of paper electrophoresis. With bromphenol blue stain the type of separation obtained with Cohn's method 10 for cord blood is the same as for adults. Albumin is concentrated almost entirely in Fraction IV + V + VI with variable amounts of alpha$_1$, alpha$_2$, and beta globulins. Noteworthy, however, is the unusually low concentration of alpha$_2$ globulin in

this fraction. The gamma globulin is almost entirely concentrated in Fraction II and only traces are found in Fraction I + III where fibrinogen, $alpha_1$, and $alpha_2$ globulins also appear but in amounts very low when compared with those of normal adults. The lipids are concentrated in Fraction IV + V + VI as $alpha_1$ globulin and in Fraction I + III as $beta_1$ globulins. As might be expected from the extremely low lipid values in cord blood, the stain with Sudan B is very faint.

DISCUSSION

A. *The concentration of protein in the fractions*

The data on protein concentrations of maternal plasma are consistent with the results of previous investigations using electrophoretic separation of the plasma proteins. The decrease in plasma albumin noted in those studies (4, 7) is masked in the Cohn fractionation since in Fraction IV + V + VI, which contains albumin, there is a considerable increase of alpha and beta globulins in the mothers' plasma. Fraction II, consisting largely of gamma globulin, is reduced in amount slightly and this is in agreement with the electrophoretic data. All previous studies have shown increases in maternal alpha and beta globulins, the influence of which is seen in the large increase in the amount of protein in Fraction I + III.

In cord plasma our results are in agreement with those of others that the total protein concentration is reduced. The reason for the reduction in protein in Fraction IV + V + VI cannot be given categorically in the absence of electrophoretic analysis of the fractions. In the study of Longsworth, Curtis, and Pembroke (4) a decrease in albumin was found in the cord plasma. In the study of Moore, DuPan, and Buxton (6) no such decrease occurred. In both studies alpha globulins were decreased. It is possible that the decrease in protein in Fraction IV + V + VI is due solely to a decrease in alpha globulins which are present in this fraction although the magnitude of the decrease suggests also some decrease in albumin concentration.

B. *The concentrations of lipids in the plasma and fractions*

The results of the study agree with others in the literature in their observation of an increased concentration both of cholesterol and of phospholipids in the maternal plasma. As in the study of Peters, Heinemann, and Man (10) the ratios of cholesterol and phospholipid in the plasma vary only within narrow limits. Examination of the fractions offers some information concerning the distribution of lipids between the two types of lipoproteins in the pregnant state. In Fraction IV + V + VI in which the lipids form a part of alpha lipoproteins, the concentrations of cholesterol and phospholipid are relatively diminished but absolutely unchanged. From this it may be inferred that at the time of delivery the pregnant woman's plasma contains alpha lipoproteins in approximately normal amounts. Our data offer no evidence from cholesterol-phospholipid ratios that the alpha lipoproteins differ in character from those in the plasma of nonpregnant women.

In Fraction I + III in which the lipids are combined chiefly if not entirely with $beta_1$ globulins as beta lipoproteins the average concentration both of cholesterol and phospholipid is increased but with the phospholipid increment sufficiently greater to cause a significant reduction of the cholesterol-phospholipid ratio of the fractions and to indicate the possibility of abnormal lipid combinations with globulins.

Cord plasma. The low concentration of cholesterol and phospholipids in fetal plasma is well illustrated by our data. Fraction IV + V + VI contains a relatively high proportion of the total lipids. Cholesterol-phospholipid ratios deviate little from normal and are consistent with those of normally constituted alpha lipoproteins. Fraction I + III with the lowest lipid concentration thus far found in our experience with human plasma reveals cholesterol-phospholipid ratios so low as to suggest either unusual lipoproteins or variant mixtures of the lipoproteins which compose the fraction in normal adults.

The depressed ratios of cholesterol to phospholipid in Fraction I + III and the relative concentration of lipids in Fraction IV + V + VI combine to explain the low cholesterol-phospholipid ratios of the unfractionated plasma.

Relation of hormones to chemical deviations in the plasma of pregnancy and the newborn. Since the pregnant woman at term is producing large amounts of estrogen, it might be suspected that

estrogen stimulation is responsible for some of the chemical disturbances that have been recorded in maternal and fetal plasma. Work in our laboratory reported elsewhere has shown that the administration of large amounts of estrogen to adults produces several predictable changes in the concentration and distribution of lipids (3). It usually causes a fall in the concentration of the total cholesterol in the plasma. It changes the distribution of cholesterol in such a manner that a large part of the total is concentrated in Fraction IV + V + VI presumably in the form of alpha lipoproteins. It causes a fall in phospholipid concentration greater than that observed for cholesterol, a change which is especially notable in Fraction I + III where the cholesterol-phospholipid ratios are definitely lower than under normal conditions.

In maternal plasma there is little to indicate that the protein-lipid pattern has been changed in a direction which would be expected from an unmodified estrogen effect. In the plasma of the infant all of the deviations expected from administration of estrogen are evident. The total cholesterol concentration is low, the percentage of total cholesterol in Fraction IV + V + VI is high, and the cholesterol-phospholipid ratios in the plasma and in Fraction I + III fall below normal. It is not impossible that some or all of these changes are related to the physical alterations in genitalia and breasts so frequently observed in newborn infants and usually attributed to temporary stimulation by estrogens. Significance may also be attached to the experience that these changes so evident at the moment of birth are lost after a few weeks of extra-uterine life.

The question raised by these comments can be answered only by further investigation. It is probable that the estrogen influence is only one of many which modify or change protein-lipid relationships during the complicated state of pregnancy and fetal development.

SUMMARY

Plasma taken from pregnant women at the time of their delivery and simultaneously from the umbilical cords of their newborn infants has been fractionated by Cohn's method 10. The whole plasma and its fractions have been analyzed for protein, cholesterol, and phospholipids.

Moving boundary and paper electrophoretic methods have been used to confirm the separation of protein components in selected cases. These determinations have shown that the protein separation is of the same type as that seen in normal adults, and that alpha lipoproteins are concentrated in Fraction IV + V + VI while beta lipoproteins accumulate in Fraction I + III. The beta lipoproteins in Fraction I + III of maternal plasma migrate electrophoretically somewhat faster than do those of normal non-pregnant women.

The study lends support to observations of previous observers that in the maternal plasma the concentration of albumin is reduced and that the concentrations of alpha and beta globulins as well as of cholesterol and phospholipids are increased; also that in cord plasma the concentrations of total protein, alpha and beta globulins, total cholesterol, and total phospholipids are less than in adult plasma.

In maternal plasma the distribution of cholesterol and phospholipids between the lipoproteins of plasma is frequently but not invariably altered so that the amount combined with alpha lipoproteins is relatively diminished and the amount combined with the proteins of Fraction I + III is relatively increased. The lipoproteins in Fraction I + III have a significantly lower than normal cholesterol-phospholipid ratio. In cord plasma the distribution of lipids between the lipoproteins deviates from that of adult plasma in such a way that a relatively greater amount is found in the fractions containing alpha lipoproteins and correspondingly less in those containing beta lipoproteins. The cholesterol-phospholipid ratios of the fraction containing beta lipoproteins are considerably reduced, and the ratios of the unfractionated plasma are far lower than those found in adults.

Although both the maternal and fetal organisms are admittedly exposed to gross stimulation of estrogen, the cholesterol-phospholipid relationships in the mother's plasma fail to show the effects observed after administration of estrogen. The infant exposed to the same stimulus displays changes which might possibly be attributed to an estrogen effect.

REFERENCES

1. Russ, E. M., Eder, H. A., and Barr, D. P., Protein-lipid relationships in human plasma. I. In normal individuals. Am. J. Med., 1951, **11**, 468.
2. Barr, D. P., Russ, E. M., and Eder, H. A., Protein-lipid relationships in human plasma. II. In atherosclerosis and related conditions. Am. J. Med., 1951, **11**, 480.
3. Barr, D. P., Russ, E. M., and Eder, H. A., Influence of estrogens on lipoproteins in atherosclerosis. Tr. A. Am. Physicians, 1952, **65**, 102.
4. Longsworth, L. G., Curtis, R. M., and Pembroke, R. H., Jr., The electrophoretic analysis of maternal and fetal plasmas and sera. J. Clin. Invest., 1945, **24**, 46.
5. Lagercrantz, C., Electrophoretic analysis of serum in pregnancy and in pregnancy toxemia. Upsala läkaref. förh., 1945, **51**, 117.
6. Moore, D. H., DuPan, R. M., and Buxton, C. L., An electrophoretic study of maternal, fetal, and infant sera. Am. J. Obst. & Gynec., 1949, **57**, 312.
7. Macy, I. G., and Mack, H. C., Physiological Changes in Plasma Proteins Characteristic of Human Reproduction. Cross Sectional and Longitudinal Electrophoretic Data for Women During and Following Uncomplicated and Complicated Pregnancies. Detroit, Children's Fund of Michigan, 1951, 170 p.
8. Von Latzka, A., Die Strukurveränderung von Blutplasmaeiweiss während der normalen Schwangerschaft. Arch. f. Gynäk., 1931, **147**, 120.
9. Boyd, E. M., and Wilson, K. M., The exchange of lipids in the umbilical circulation at birth. J. Clin. Invest., 1935, **14**, 7.
10. Peters, J. P., Heinemann, M., and Man, E. B., The lipids of serum in pregnancy. J. Clin. Invest., 1951, **30**, 388.
11. Boyd, E. M., Lipid composition of blood of newborn infants. Am. J. Dis. Child., 1936, **52**, 1319.
12. Goldwater, W. H., and Stetten, D., Jr., Studies in fetal metabolism. J. Biol. Chem., 1947, **169**, 723.
13. Smith, C. A., The Physiology of the Newborn Infant. 2nd ed., Springfield, Charles C Thomas, 1951, 348 p.
14. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K., and Uroma, E., A system for the separation of the components of human blood: Quantitative procedures for the separation of the protein components of human plasma. J. Am. Chem. Soc., 1950, **72**, 465.
15. McDonald, H. J., Ionography: A new frontier in electrophoresis. J. Chem. Educat., 1952, **29**, 428.
16. Flynn, F. V., and de Mayo, P., Micro-electrophoresis of protein on filter-paper. Lancet, 1951, **2**, 235.
17. Cremer, H., and Tiselius, A., Elektrophorese von Eiweiss in Filtrier-papier. Biochem. Ztschr., 1950, **320**, 273.
18. Goa, J., A simplified method for the separation and quantitative determination of serum proteins by paper electrophoresis. Scandinav. J. Clin. & Lab. Invest., 1951, **3**, 236.
19. Swahn, B., A method for localization and determination of serum lipids after electrophoretical separation on filter paper. Scandinav. J. Clin. & Lab. Invest., 1952, **4**, 98.
20. Lever, W. F., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K., and Schultz, E. L., Chemical, clinical, and immunological studies on the products of human plasma fractionation. XL. Quantitative separation and determination of the protein components in small amounts of normal human plasma. J. Clin. Invest., 1951, **30**, 99.

THE INTRAVENOUS INJECTION OF BOVINE CRYSTALLINE PANCREATIC DESOXYRIBONUCLEASE INTO PATIENTS [1]

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In connection with investigations of streptococcal desoxyribonuclease contained in Varidase®,[2] comparative observations have included the intravenous administration of crystalline pancreatic desoxyribonuclease in patients (1).

Crystalline pancreatic desoxyribonuclease (referred to throughout this article as PD) has been isolated from beef pancreas and its general properties determined (2–4). The crystalline material [3] made according to the methods of Kunitz, has been employed in this study.

The results to be described center primarily around the intravenous injection of PD into patients. In addition, observations have been made on the renal excretion and clearance of PD, its diffusion into various extravascular areas and the effect of intrathecal injections in adults and children with and without meningitis. Extensive clinical and laboratory examinations have been made for evidence of toxicity.

The categories of the studies may be listed as: a) Toxicity studies following intravenous and intrathecal injections; b) blood levels following intravenous infusions; c) "naturally" occurring inhibitor for PD (in the general circulation); d) renal excretion and clearance; e) diffusion into the peritoneal and pleural fluids, wound exudates, bronchial secretions and cerebro-spinal fluid; and f) the development of specific antibodies.

A discussion of the potential therapeutic implications of PD follows.

[1] This study was supported by grants from the National Institutes of Health, U. S. Department of Health, Education, and Welfare, and Lederle Laboratory Division, American Cyanamid Company.

[2] Streptokinase-Streptodornase, Varidase®, Lederle Laboratories, Pearl River, N. Y.

[3] Supplied by Worthington Chemical Co., Freehold, New Jersey.

MATERIALS AND METHODS

Enzyme substrate

A 0.15 per cent solution of purified calf thymus desoxyribonucleic acid [4] (DNA) was prepared by adding the dried sodium thymonucleate to M/40 veronal buffer pH 7.5, and agitating in a shaking machine until the DNA went into solution. After shaking, the solution was heated at 56° C. for 5 to 25 hours until residual depolymerase activity was destroyed. Further heating was used (*a*) to reduce the viscosity to a specific viscosity of about 5 to 7.0 (4.0 centipoise to 5.6 centipoise) and (*b*) to inhibit the "reactivity" of the DNA. Inasmuch as the assay value for a standard control solution of desoxyribonuclease could be decreased by prolonged heating of the DNA substrate used for its assay, it seemed likely that the "reactivity" of the DNA was controlled by this heating. This decrease occurred without an apparent change in the viscosity of the DNA. In order to obtain consistent and reproducible results with this enzyme assay, it proved to be obligatory to control these three variables: the stability, viscosity, and "reactivity" of the substrate. Mg^{++} was added to the DNA solution after heating to a final concentration of .003 M.

Desoxyribonuclease assay

The assay for desoxyribonuclease activity (5) was modified as follows: *a*) 0.1 ml. or 0.2 ml. of sample was used interchangeably as required, for optimum enzyme concentration in the assay; *b*) recovery experiments with PD in neopeptone buffer, urine, and cerebro-spinal fluid indicated a linear relationship between enzyme concentration and decrease in viscosity of the DNA substrate from 0.2 to 0.8 viscosity units in 10 minutes. However, PD in inhibitor-free sera could not be measured accurately unless the reduction in viscosity was at least 0.4 viscosity units in 10 minutes; *c*) the assay was performed for as long as necessary (15 to 90 minutes) to obtain a readily measurable, sustained, *linear* fall in viscosity.

Assays on PD in urine were complicated by the adsorption of small amounts of PD on phosphate crystals. Therefore, prior to assay, crystal formation in the samples of urine was minimized by incubation in a 35° C.

[4] The desoxyribonucleic acid was supplied by Lederle Laboratories, Pearl River, N. Y.

waterbath for 5 to 10 minutes and by adjusting the pH to 6.5 with N/10 HCl.

Neopeptone buffer (6) was used to dilute the samples containing PD for assay. A 1 per cent solution of human serum albumin [5] in physiological saline was used to dilute the enzyme prior to injection. Saline-albumin was found to be an excellent stabilizing agent for dilute enzyme preparations (20 micrograms per ml.)

PD inhibitor

One-tenth of one ml. of a standard solution containing 80 units of PD per ml. was added to 0.9 ml. of fresh serum (or serum stored when fresh at −20° C.) to produce a final concentration of 8 units of PD per ml. of serum. The mixture was incubated at room temperature for 15 minutes, with gentle mixing by inversion every 5 minutes. The mixture was then assayed and a recovery of less than 7.7 units of PD was considered evidence of inhibition. A further check on the method was made possible because the reversible, immunologically non-specific inhibition was removed by heating the mixture in a water bath at 35° C. for 5 to 10 hours. At the end of the heating period, recovery of the original 8 units from the sera was obtained.

PD antibody

The viscosimetric antibody determination was performed as previously described for SD (7), except for the fact that the sera were heated at 56° C. for 2 to 4 hours prior to incubation with the enzyme solution.

Proteolytic activity

Fibrinolysis and fibrinogenolysis were used to estimate the proteolytic activity of the PD. Various concentrations of PD, from 0.1 mgm. per ml. to 100 mgm. per ml. were mixed with a solution of fibrinogen and clotted with thrombin to produce a standard clot (5, 8). Lysis of the clots in the tubes with PD before those in the controls was considered evidence of proteolytic activity. The control clots were stable for at least 24 hours. Fibrinogenolysis was determined by incubating the fibrinogen with comparable amounts of PD for four hours, after which (*a*) thrombin was added to produce a clot, or (*b*) 10 per cent trichloracetic acid was added to facilitate testing for acid-soluble tyrosine in the supernatant. Absence of clot formation or an increase in acid-soluble tyrosine in the supernatant indicated fibrinogenolysis. Tyrosine was determined as previously described (8).

Feulgen stain

The Feulgen stain was carried out on samples that were smeared on carefully washed slides and air-dried without fixation (9).

[5] Normal serum albumin (human) salt-poor, U.S.P., Cutter Laboratories, Berkeley, California.

Samples

Merthiolate,[6] 1:10,000 was used as a preservative in all samples unless otherwise indicated.

Serum: Fresh, whole blood was allowed to clot in new plastic [7] centrifuge tubes, centrifuged within 1 hour and the decanted serum stored at −20° C.

Plasma: Plasma was obtained using Amberlite 1R-100 [8] and Amberlite 1R-120,[8] (10), respectively. This was done because citrate or oxalate inhibits PD (6, 11). Furthermore, preliminary studies in this laboratory have shown that relatively low concentrations of heparin [9] also inhibit PD.

Sputum: Sputum samples were centrifuged at 5,000 to 10,000 rpm and the supernatant used for assay. When high viscosity sputum made pipetting of the supernatant inaccurate, the sputum was mixed thoroughly, diluted 1:2 with saline, then centrifuged, and the supernatant used for assay.

Clinical-pathological laboratory determinations

Conventional methods were used to determine the red blood count, white blood count and differential on whole blood and spinal fluid, as well as the hemoglobin and hematocrit, the bleeding time (Duke method), coagulation time (Lee-White) and the prothrombin time (Quick method with Simplastin [10]) (12, 13). Preparations for the demonstration of the Lupus erythematosus cell (L–E cell) were made by the heparin (14) and defibrination techniques (15).

The total protein content of the cerebro-spinal fluid was determined by a modified tyrosine method (8), and by a modified sulfosalicylic acid method (16) and checked by the Kjeldahl technique (17). The cerebro-spinal fluid sugar was analyzed by the Folin-Wu method (18).

RESULTS

Toxicity studies following intravenous injection of PD into patients

Crystalline pancreatic desoxyribonuclease (PD) has been injected in 65 adults and 45 children.[11] The intravenous dosage was successively increased

[6] Merthiolate (Thimerosal), Eli Lilly and Co., Indianapolis, Indiana.

[7] Lusteroid Container Corp., Inc., Maplewood, New Jersey.

[8] Amberlite 1R-100 and Amberlite 1R-120, Rohm and Haas Co., Philadelphia, Pa.

[9] Heparin, sodium salt, Connaught Medical Research Laboratories, Toronto, Ontario, Canada.

[10] Simplastin, Chilcott Laboratories, Morris Plains, New Jersey.

[11] The studies in children were made on the Pediatric Service of Dr. L. Emmett Holt. The interest and advice of Dr. Holt and Dr. Edith Lincoln have proved invaluable.

from 10 units to 2,000,000 units per injection. Sixteen patients received more than 100,000 units but less than 1 million, and 41 patients received 1,000,000 to 3,000,000 units. There was no evidence of systemic toxicity in the patients receiving the PD by intravenous injections or by other parenteral routes, including intrathecal. The criteria for toxicity were based upon symptomatology, temperature, pulse, blood pressure, urine analysis, as well as the red blood count, hemoglobin, hematocrit, white blood count and differential, and the bleeding time, coagulation time, prothrombin time, and "Lupus erythematosus (L. E.) cell" preparations.

Special attention was given to the speed of injection in order to prevent a non-specific foreign protein reaction. The injection time was varied from 2 minutes to several hours at each dosage level. No reactions occurred.

The crystalline PD was also tested for contamination by other potentially toxic proteins, notably trypsin inasmuch as Kunitz has shown that trypsinogen and PD can be precipitated from an acid extract of beef pancreas (3) with 20 and 40 per cent saturated ammonium sulfate, respectively. Several preparations of PD were tested for tryptic activity by incubation in various concentrations with fibrin and fibrinogen. No measurable proteolytic activity was seen. Since trypsin accelerates prothrombin conversion to thrombin and has been observed to shorten the coagulation time and cause thrombosis (19, 20) *in vivo,* freshly drawn whole blood was taken in a siliconed syringe,[12] measured by siliconed pipettes, and mixed in a Lee-White tube with 0.1 ml. of various concentrations of PD. No acceleration in the clotting time was observed. Furthermore, no local thromboses have been noted following the injection of PD intravenously.

Since it has been suggested that the L.E. cell phenomenon may be potentiated by increased serum desoxyribonuclease or destruction of an intracellular inhibitor (21, 22), L.E. cell preparations were carried out at various time intervals following the intravenous injection of 1,000,000 units of PD, by both the heparin and clot methods on blood from normal individuals who had high, medium, and low serum levels of PD. There was no evidence of L.E. cell transformation in these *in vivo* studies, thus corroborating the earlier *in vitro* work of Kurnick, Pariser, Schwartz, Lee, and Irvine (23).

[12] DC 200, Dow-Corning Corp., Midland, Mich.

Toxicity studies following the intrathecal injection of PD into patients

Intrathecal injections were made in normal individuals and in patients with meningitis in amounts ranging from 10 units to 500,000 units in a single injection. Approximately 180 injections have been administered to 38 patients; 16 of these were children. About 80 per cent of the injections ranged from 25,000 to 50,000 units per injection. The tests for toxicity were those enumerated above, and in addition, the cerebro-spinal fluid total-cell count, white blood count, differential, total protein and sugar. Estimation of the cerebro-spinal fluid pressure and the Queckenstadt maneuver were also routine.

Fifteen normal adults were given PD intrathecally from six different batches of PD. Two received 25,000 units of PD intrathecally from lots A and B, respectively. The only reaction was a rise in the white blood count of 10 to 15 cells in the cerebro-spinal fluid, largely lymphocytes. Three patients received 50,000 units from lot C (processed in a different manner from the other batches). Each of the three patients had a mild temperature rise of 1 to 2° F. with mild headache, a rise in cerebro-spinal fluid white blood cells to nearly 2,000 cells and a rise in cerebro-spinal fluid pressure to 200 to 250 mm. of water. The symptoms disappeared in two days and the cerebro-spinal fluid returned to normal in 3 to 4 days. This batch of PD was subsequently discarded. Two patients received 50,000 units of lot E, and three patients received 50,000 units of lot F without apparent toxic effect other than a transient increase in the cerebrospinal fluid white blood cell count to 30 to 50 cells (90 per cent lymphocytes).

One vial of lot D had been mixed several weeks before it was injected and contained a very small stringy precipitate. About 8 to 10 hours following the intrathecal injection of lot D in two patients, each of them had a one degree rise in temperature with a sharp rise in white blood cells in the cerebro-spinal fluid (to nearly 1,000 cells, 95

per cent polys), and a 1 + Pandy reaction. The cerebro-spinal fluid returned to normal in 3 days. Two additional patients received freshly mixed preparations of the same lot D. No reaction occurred except a slight rise in the white blood cells of the cerebro-spinal fluid to 10 to 15 lymphocytes. A vial of freshly mixed preparation of the same lot D above was shaken vigorously until a small stringy precipitate was produced, and the contents of the vial were injected intrathecally. Once again, a mild febrile reaction occurred with a sharp rise in the white blood cells of the cerebro-spinal fluid. The enzyme titer of each of the vials of lot D, at the time of injection, was found to be the same, irrespective of whether the samples were fresh, old, or agitated. The contents of each of the vials was cultured and was found to be sterile. Since the toxic reaction occurred when the stringy precipitate was seen, and did not occur when the same lot free of precipitate was used, the toxicity was assumed to be due to denatured protein.

Various lots of PD (except lot C) were given intrathecally in repeated injections of 50,000 to 100,000 units to 15 patients with pyogenic and 3 patients with tuberculous meningitis. Toxic reactions were not observed. The pyogenic meningitides included three cases due to *H. influenzae,* three cases due to *D. pneumoniae,* seven cases due to meningococcus, and two cases due to staphylococcus aureus. Occasionally, the cerebro-spinal fluid pressure in a normal, or meningitic patient has been found to be low following the injection of the enzymes. This effect was transient and the reasons have not been apparent. A definite increase in pressure, possibly attributable to the enzyme has been seen just once. A six months old infant with E. coli meningitis was given 200,000 units of PD intrathecally, 300,000 units the following day, 400,000 units the next, and 500,000

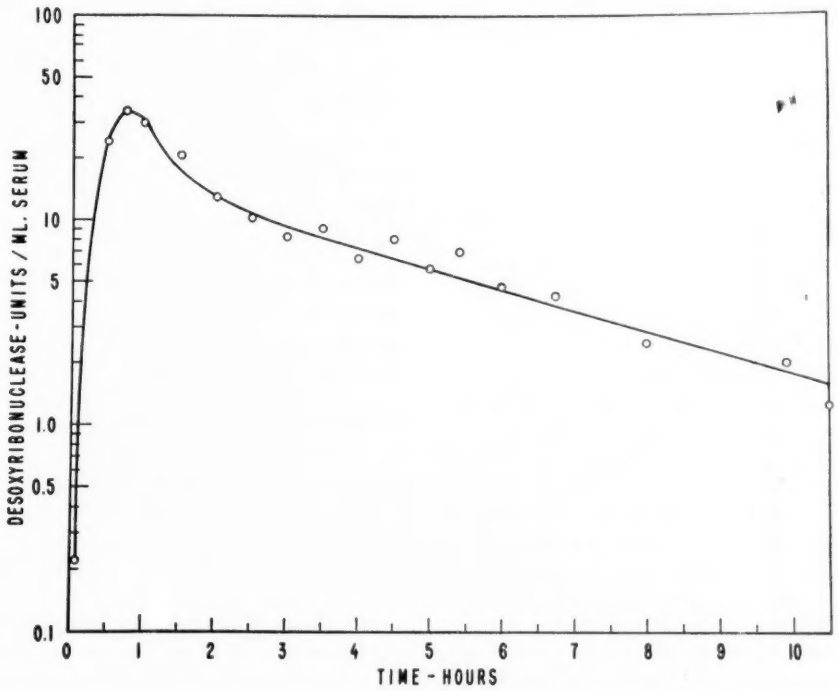

FIG. 1. SEMI-LOG PLOT OF THE AVERAGE SERUM LEVELS OF PD FOR EIGHT PATIENTS FOLLOWING THE RAPID INTRAVENOUS INFUSION OF 1,000,000 UNITS OF PD

The initial level represents an average of the endogenous serum levels.

units on the fourth day. The cerebro-spinal fluid pressure rose transiently to about 300 mm. of water. On the other hand, 100,000 units of PD were administered daily, intrathecally for nearly 14 days to another child (two years old), with tuberculous meningitis, without an increase in cerebro-spinal fluid pressure.

Six patients received repeated injections of large amounts of PD over a prolonged period. Successive injections were given intravenously and/or intrathecally for periods ranging from two days to two months, making a total of more than 60,000,000 units in 83 intravenous injections, 109 intrathecal injections and two endobronchial instillations. Two children with tuberculous meningitis received very large amounts of PD. One received about 23,000,000 units in 27 intravenous injections and 2,000,000 units in 18 lumbar (intrathecal) and 25 intra-ventricular injections over a period of one and one-half months. The other was given more than 11,000,000 units in 24 intravenous injections and nearly 3,000,000 units in 24 lumbar and 20 intra-ventricular injections over a comparable period. There was no systemic or local toxicity evident.

Blood levels of PD following intravenous injections

Serial blood determinations were made on 20 patients following a single injection of 1,000,000 units of PD. The average curve for eight patients (Figure 1) indicates a serum level of 30 units of PD per ml. about one hour after the termination of the infusion, with a range of 18 to 50 units.

The maximum serum level following intravenous injection fell progressively for 8 to 12 hours, when it tended to level off. A level of 0.5 to 1.0 unit of desoxyribonuclease was found in the serum for 24 to 48 hours following the injection. A low initial level of desoxyribonuclease activity was found in the serum, apparently due to endogenous DNase as previously described (24–26). The level of endogenous DNase found in normal individuals by the technique used was approximately 0.05 unit.

The naturally occurring inhibitor for PD in the circulating blood was found to be destroyed by heating at 35° C. for 5 to 10 hours. PD remained stable during this heating process. Consequently, when serum from a patient following

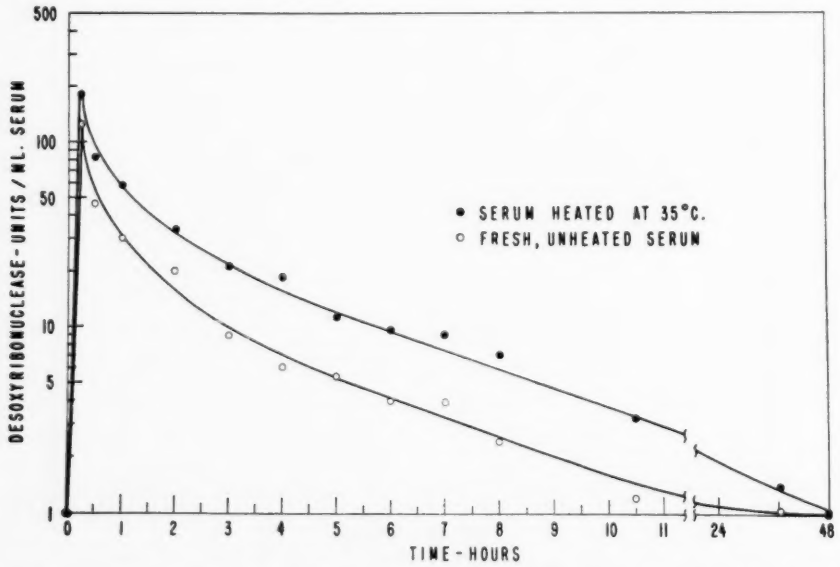

FIG. 2. SEMI-LOG PLOT OF THE SERUM LEVELS OF PD AS DETERMINED ON HEATED AND UNHEATED SERA BEFORE, DURING, AND AFTER THE RAPID INTRAVENOUS INFUSION OF 1,000,000 UNITS OF PD IN A PATIENT

the intravenous injection of PD was heated to 35° C. for 5 to 10 hours the assay of PD was 30 to 60 per cent higher than for the unheated sera. The specific differences between measurements made on heated and unheated samples derived from a single patient are given in Figure 2.

Assays for PD on the unheated sera averaged about 45 per cent of the values for assays on the heated sera indicating about 55 per cent inhibition of the PD in the serum of this patient. This amount of inhibitor was corroborated by inhibitor and recovery studies performed on control samples *in vitro*.

The causes of the progressive decline in blood level of PD and the rate of this fall (Figure 2) are probably referable to several factors. Kurnick has described the sequestration, excretion, and metabolism of endogenous rat desoxyribonuclease by the intact rat liver (*27*). Observations of excretion and diffusion, to be subsequently described, indicate other routes by which PD may leave the general circulation.

"Naturally" occurring inhibitor

When sera containing added PD was heated for about 8 hours at 35° C., complete recovery of the PD occurred. Recovery of the added PD from a few samples was accomplished after only 2 to 3 hours of heating at 35° C., about 8 hours sufficed for most samples, while a few required as long as 10 to 15 hours. The heat lability of the PD inhibitor varied from patient to patient, and from specimen to specimen taken at one-half-hour intervals from the same patient. The reasons for this variability are not known. The concentration and type of cation in the plasma may play a role in stabilizing the inhibitor inasmuch as the inhibitor in plasma that was prepared by the ion-exchange method was resistant to heat inactivation for 10 to 15 hours, while the inhibitor in serum controls drawn at the same time was heat inactivated in two and one-half to three hours.

In the experiment detailed in Figure 3, 0.1 ml. of a neopeptone buffer solution containing 8.0 units of PD was added to 0.9 ml. of fresh, undiluted serum. The mixture was agitated gently and allowed to stand at room temperature for 15 minutes after which a sample was drawn for assay. The mixture was then placed in the 35° C. waterbath and incubated for 5 hours following which another sample was drawn for assay. In the assay on the unheated serum, the inhibition as indicated by the viscosity-time plot was most apparent in the first 15 minutes of the curve. During the next 5 to 10 minutes the inhibition gradually diminished and thereafter inhibition was nearly absent. Additional data suggest that the decrease in inhibition with time was due to dilution of the inhibitor by the large volume of substrate used in the assay with subsequent dissociation of a reversible inhibitor-enzyme complex. This was also emphasized by the fact that the slope of the second portion of the curve for the unheated

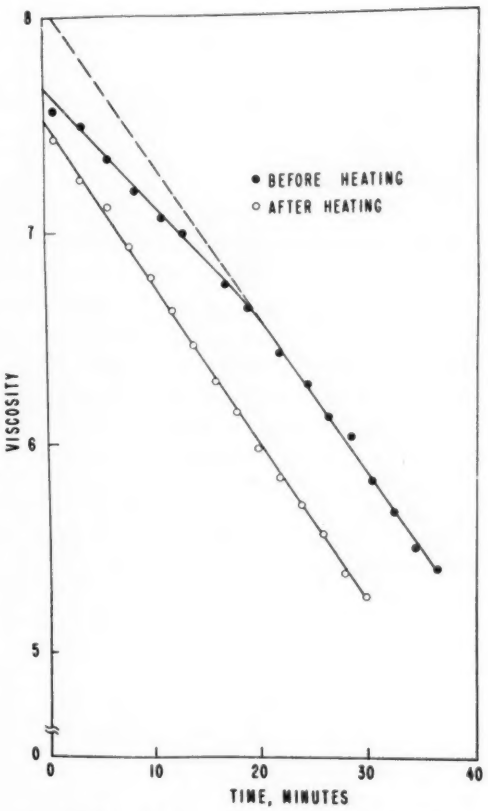

FIG. 3. HEAT INACTIVATION OF SERUM INHIBITOR FOR PD

The slope of the second part of the curve of the unheated serum was extrapolated to the Y axis for purposes of comparison.

TABLE I

Inactivation of serum inhibitor for PD by heat

Serum	DNase units / ml. serum *units*	Inhibition %
Before heating*	4.6	43
Heated 3 hours at 35° C.	5.9	26
Heated 6 hours at 35° C.	6.4	20
Heated 9 hours at 35° C.	7.8	3

* Eight units of PD were added to each ml. of serum at the beginning of the experiment.

sera was nearly parallel to the slope of the curve of the heated sera. In addition, the curve seen with fresh serum showing inhibition (Figure 3), was replaced by a straight line, without evidence of inhibition, after the sera was heated at 35° C. for 5 hours. The recovery experiment in Table I shows the inactivation of serum inhibitor over a nine-hour period. Following the addition of eight units of PD to 0.9 ml. of fresh serum, assays were performed on the mixture at 0 time and after 3, 6, and 9 hours incubation at 35° C. As the heating was continued, the inhibitor was inactivated, and the per cent inhibition decreased gradually from 43 per cent to 3 per cent in 9 hours.

The inhibitor was destroyed by heating the sera at 56° for 5 minutes before addition of the enzyme, by storage in the refrigerator for several months, storage at room temperature for several days, extremes of pH, and the addition of trypsin.[13] The inhibitor was maximally precipitated by ammonium sulfate at 40 per cent saturation and was not affected by dialysis, quick freezing and thawing, or storage at − 20° C. for long periods of time. Because it was heat labile, non-dialyzable, and destroyed by trypsin the inhibitor was thought to be a protein or part of a protein complex. The inhibitor appeared to differ from those previously shown to be derived from white blood cells (28–30) because it was present in equal

[13] Crystalline trypsin, Armour Laboratories.

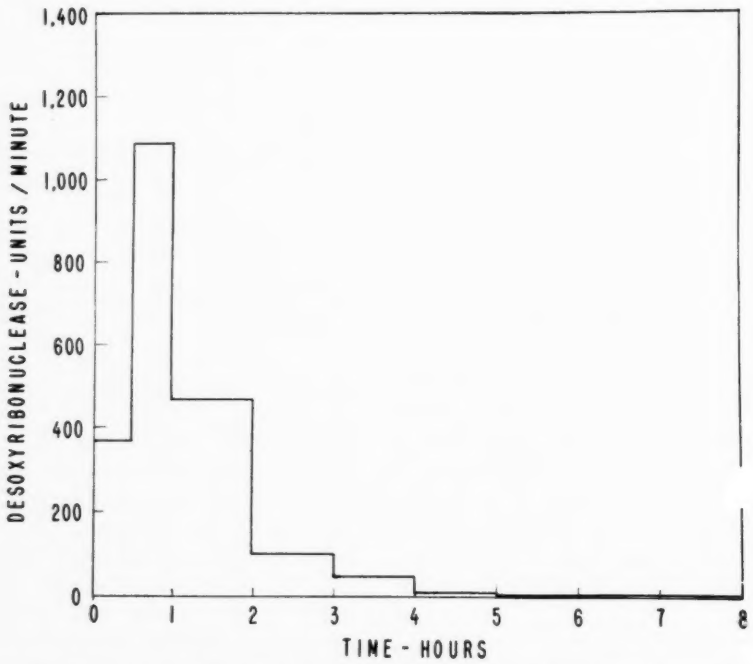

FIG. 4. URINARY EXCRETION OF PD FOLLOWING THE INTRAVENOUS INJECTION OF 1,000,000 UNITS OVER A PERIOD OF THIRTY MINUTES IN A PATIENT WITHOUT RENAL DISEASE

The injection began at 0 time.

Eighty thousand five hundred units of PD were excreted in four hours.

amounts in serum contaminated by cellular proteins, plasma free of white blood cells, and plasma-white cell concentrates. The serum for this experiment was derived in the conventional manner from whole blood and inevitably contained soluble proteins from traumatized white blood cells; the plasma was decalcified by ion-exchange resin and centrifuged gently for at least one-half hour until it was free of white blood cells. The plasma-white cell concentrates were obtained by differential centrifugation of the resin-treated plasma and from the heparinized blood of patients with infection whose white blood cell counts were above 30,000 per cu. mm. The concentrates were placed in the ice-box for three to four days in order to permit autolysis of the white blood cells. Release of the previously described inhibitor into the plasma supernatant was expected to accompany autolysis of the white blood cells, but no increase in non-specific inhibition was noted.

These experiments suggested that the inhibitor in human serum is not concentrated in the white blood cells (28–30). On the other hand, the properties of the inhibitor in *human serum* are consistent, in some respects, with those of a reversible inhibitor occurring in the soluble supernatant from *tissue* homogenates derived from the crop gland of brooding pigeons and bull testes (31–33).

Renal excretion and clearance

When PD was given intravenously, large amounts were excreted in the urine without demonstrable proteinuria or hematuria.

Thus, in a representative instance (Figure 4), during the intravenous infusion of 1,000,000 units of PD, the excretion of desoxyribonuclease in the urine rose from a pre-injection level of 15 units per minute to 360 units per minute in thirty minutes. One-half hour after the infusion was completed the urinary excretion of PD reached a maximum of 1,090 units per minute and fell, thereafter, to approximately control levels within the ensuing 5 to 6 hours.

When a total dose of 10,000 to 1,000,000 units was given in thirty minutes or less, 5 to 10 per cent of the injected dose was regularly recovered in the urine. However, when the same dosage was given over four hours, as much as 20 per cent of the injected dose was excreted in the urine.

Inasmuch as a majority of these determinations were made during a falling plasma level of PD, several experiments were conducted by the constant infusion technique with a sustained plasma level.

In this experiment (Figure 5) 100,000 units of PD were injected intravenously at zero time and 800,000 units were infused at a constant rate of 5,000 units per minute for 160 minutes. A rea-

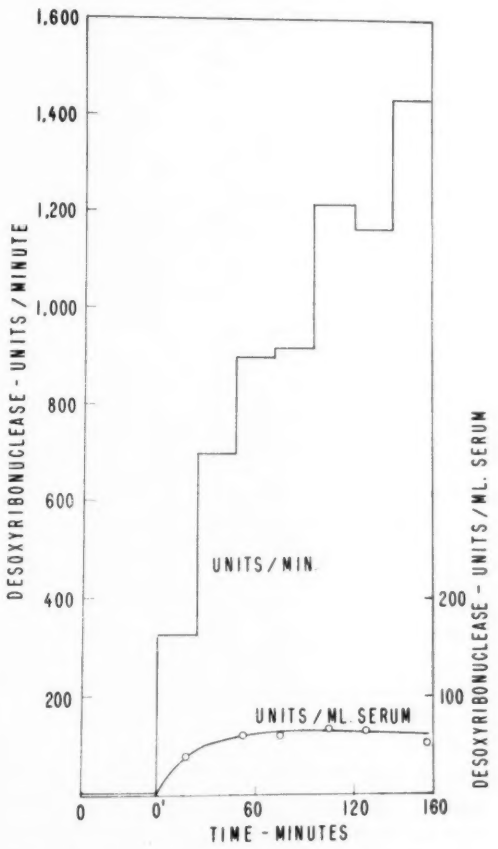

FIG. 5. URINARY EXCRETION OF PD BEFORE AND DURING THE CONSTANT INTRAVENOUS INFUSION OF 800,000 UNITS OF PD OVER A PERIOD OF 160 MINUTES IN A PATIENT WITHOUT RENAL DISEASE

0 — 0′ represents a control period. At 0′ the constant intravenous infusion was started at a rate of 5,000 units per minute and a priming dose of 100,000 units was injected within 2 minutes.

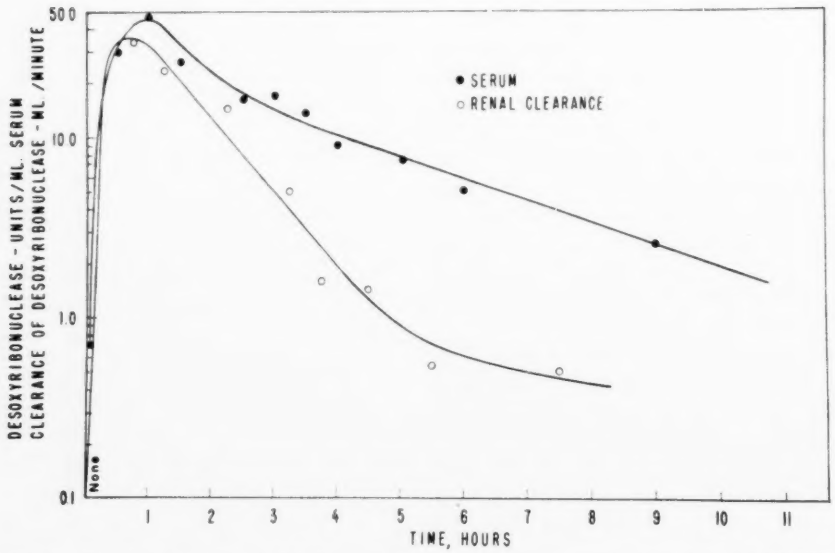

FIG. 6. RENAL CLEARANCE OF PD FOLLOWING A SINGLE INTRAVENOUS INJECTION OF 1,000,000 UNITS IN A PATIENT WITHOUT RENAL DISEASE

sonably constant level of PD in the serum was reached in 50 minutes. In spite of this, the urinary excretion rose continuously during the entire infusion period.

The average of 12 clearance periods obtained on four patients without renal disease who had received constant infusions of PD was 17 ml. per minute, while the range was 13 to 27 ml. per minute. For purposes of comparison the clearance of a protein of a similar molecular size, albumin, in patients with nephrosis has been reported to be 0.5 to 1.0 ml. per minute (34).

Renal clearances of PD were obtained in 11 other patients who were given a single injection of 1,000,000 units of PD over a relatively short period of time without a sustaining infusion. In a typical experiment (Figure 6) the clearance periods were run before, during, and after the infusion of PD, and may be seen to rise and fall with the level of PD in the serum.

However, comparing the clearance data on a large number of patients, there seemed to be little or no correlation between the clearance of PD and the level of PD in the serum, or the urine flow.

Glomerular filtration, tubular reabsorption and/or tubular excretion were considered as possible mechanisms for the excretion of PD.

PD is a protein of the albumin type with a molecular weight of approximately 60,000 (3). It is recognized that crystalline proteins like PD may not be completely uniform in molecular size and configuration. Nevertheless, the high molecular weight of PD and the low clearances, compared to inulin, mitigate against simple glomerular filtration. Further, if some glomerular filtration did occur, the rising excretory rate of PD at a constant serum level would make a constant reabsorptive process unlikely, but would not rule out a variable rate of reabsorption by the tubules. It is possible that PD may be reversibly bound by the serum proteins, thus complicating the interpretation of the mechanism or mechanisms of renal excretion. It is also possible that PD may be actively excreted by the renal tubular cells, and the rate of excretion may, in turn, depend upon the duration of the infusion and the subsequent "loading" of the tubular cells. Further studies on the mechanism of renal excretion are in progress.

In view of the large amounts of PD found to be excreted in the urine, 1,000,000 units of PD were given intravenously to an 86 year old female, Z. P., with profoundly purulent urine due to cystitis. No antibiotics were given. The photograph in Figure 7 shows the urine before the intravenous injection of PD, 7 hours after, and 24

hours post-injection, and illustrates the striking effect of high concentrations of PD in the urine on desoxyribose nucleoprotein present in purulent urine and in the urinary tract. The effect in this patient was transitory and the exudate started to return in 24 hours. The potentialities of the treatment of urinary infections will be subsequently considered in the discussion.

Endogenous desoxyribonuclease in the urine of man. Desoxyribonuclease was found to be present in samples of normal urine in amounts ranging from 0.5 to 15 units per ml. of urine. In the present study the urinary PD was not considered to be of bovine origin unless the concentration in the urine was at least 3 to 5 times the pre-injection level.

Diffusion of intravenously injected PD into serous cavities and wounds

1. *Diffusion of PD into ascitic fluid.* Five patients with ascites due to cirrhosis of the liver were studied.

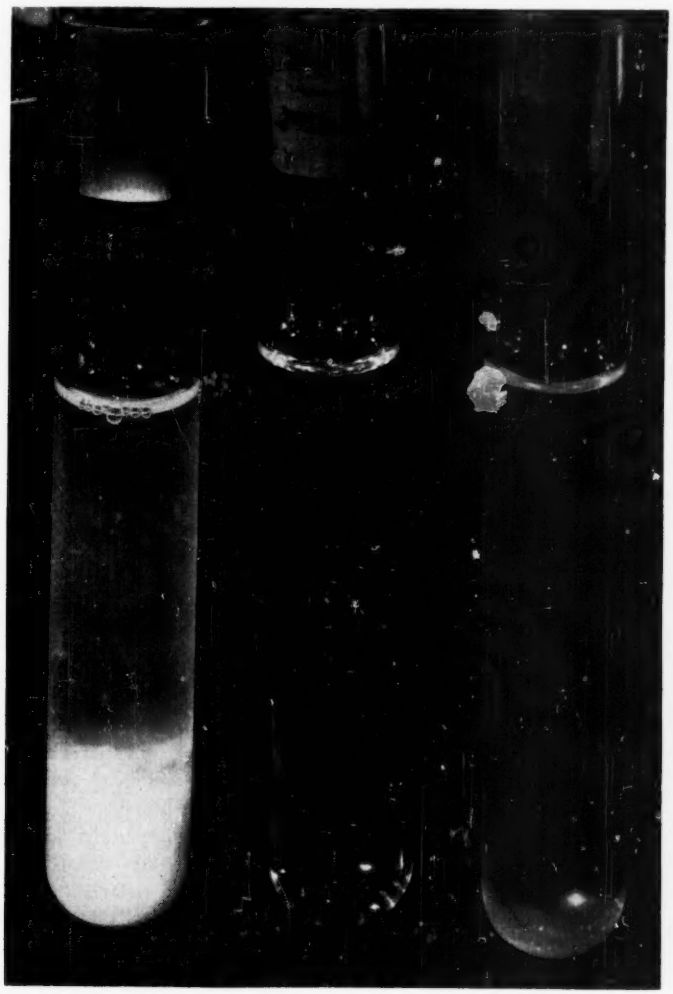

FIG. 7. EFFECT OF A SINGLE INTRAVENOUS INJECTION OF 1,000,000 UNITS OF PD IN A PATIENT WITH PURULENT CYSTITIS

The urine samples were taken before the injection of PD, 7 hours after, and 24 hours post-injection.

In a representative patient given a single intravenous injection of 1,000,000 units of PD (Figure 8) the abrupt rise and fall of PD in the serum is paralleled by a similar, but lesser rise and fall in the amount of PD in the ascitic fluid. The rise of PD in the ascitic fluid usually started about 30 minutes following the intravenous injection, reached a maximum in 3 hours, which varied in different patients studied from 0.2 to 1.0 units per ml. and persisted at lower levels (0.1 to 0.2 units per ml.) for at least 24 hours. One patient showed a persistent low level of PD (0.2 units) in the ascitic fluid for 5 days without measurable amounts in the serum.

2. Diffusion of PD into pleural effusions. Four patients with pleural effusions have been studied.

In a representative patient with tuberculous pleural effusion the intravenous injection of one million units of PD resulted in a rise and then fall in the serum level with a similar rise of PD in the pleural fluid (Figure 9). However, fluctuations occurred in the level of PD in pleural fluid obtained from two different sites which were probably separated by pleural adhesions, inasmuch as there were also marked differences in the character of the fluid obtained from them. Eight hours after the infusion, the level of PD in the pleural fluid was higher than the blood level (Figure 9).

The maximum level of PD in the pleural fluid varied considerably, and ranged from 0.1 to 1.9 units per ml. in the four patients studied. The amount of PD recoverable from the pleural fluid, as well as the duration over which PD was recoverable seemed to vary inversely with the amount of purulent exudate present. In two cases with relatively small amounts of exudate, 0.1 to 0.2 units of PD per ml. were found in the pleural fluid 24 hours after the intravenous injection. In the other two patients with large amounts of purulent pleural exudate, no PD was evident 24 hours post-injection. This was thought to be due to the large amounts of substrate (DNA) in the purulent exudates.

One patient with a 3-week history of severe cough and chest pain had, on admission to the hospital, a pneumococcal pneumonia, Type III, complicated by an empyema and a fever of 102°. The empyema fluid was thick, green and markedly purulent. Penicillin and 1,000,000 units of

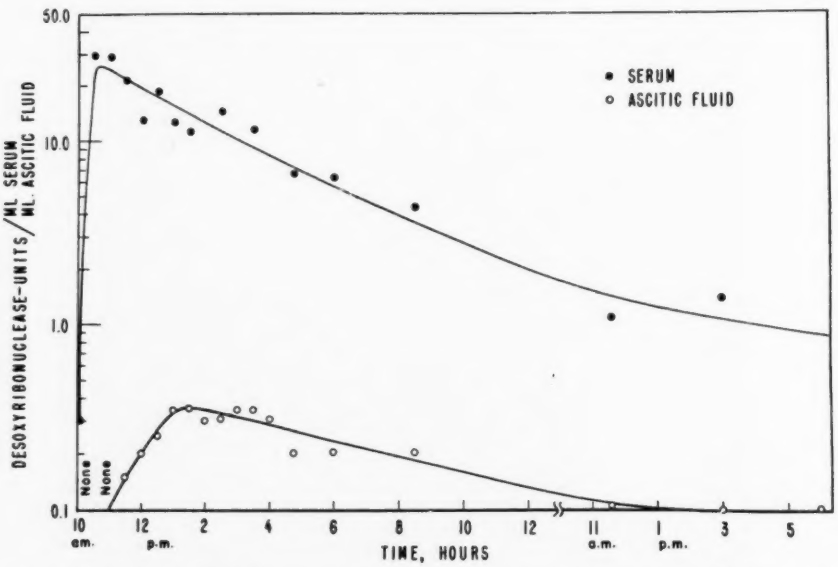

FIG. 8. SEMI-LOG PLOT SHOWING DIFFUSION OF PD INTO ASCITIC FLUID FOLLOWING A SINGLE INTRAVENOUS INJECTION OF 1,000,000 UNITS OF PD IN 10 MINUTES IN A PATIENT WITH LAENNEC'S CIRRHOSIS

The injection was started at 10:00 A.M.

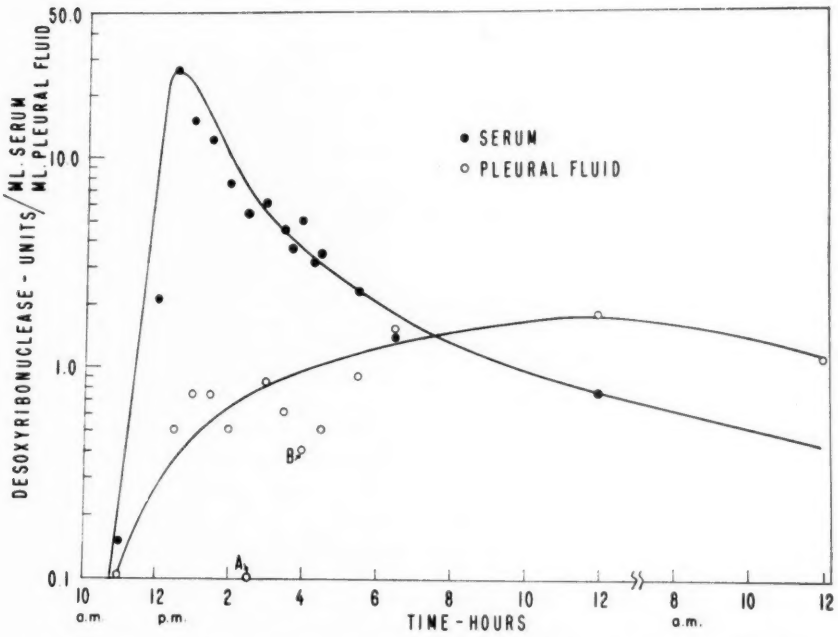

FIG. 9. SEMI-LOG PLOT SHOWING DIFFUSION OF PD INTO PLEURAL FLUID FOLLOWING A SINGLE INTRAVENOUS INJECTION OF 1,000,000 UNITS OF PD IN 10 MINUTES IN A PATIENT WITH TUBERCULOUS PLEURAL EFFUSION

The injection was started at 11:30 A.M.
A and B represent two different empyema pockets.

PD were given intravenously. Within 48 hours the patient's clinical condition improved markedly, his temperature returned to normal, and the empyema fluid became thinner but still contained many white blood cells. Three days later the empyema fluid was thin, straw-colored, sp. gr. 1012, with a marked reduction in white blood cells. The findings suggest a correlation between the diffusion of PD into the empyema and rapid clinical improvement.

3. *Diffusion of PD into wound exudates.* Two patients who had fresh granulating wounds without evidence of suppurative infection or excessive diapedesis of red blood cells were studied. One patient had a leg ulcer, the other a deep nephrectomy wound. The wounds were sealed with a rubber dam using tincture of benzoin as a rubber cement on the skin and rubber dam. Samples of exudate were taken by washing the wound surface with 1 ml. of saline before and 8 hours after the intravenous injection of 1,000,000 units of PD. No desoxyribonuclease was present in the washings from the leg ulcer before injection, while 0.1 of a unit per ml. was found before injection in the drainage from the nephrectomy wound. About 8 hours post-injection there was 0.6 of a unit per ml. in the washings from the leg ulcer and 1.6 units per ml. in the washings from the nephrectomy wound.

The diffusion of intravenously injected PD into tissue fluids was demonstrated by measurements made on edema fluid obtained by Southey tubes inserted into the legs of a patient with cardiac failure. The level of PD in the edema fluid started to rise about one-half hour after the rapid injection of 1,000,000 units of PD, reached a maximum of 0.6 of a unit in about 3 hours, and fell to normal (0.05 units) in about 10 hours.

4. *Diffusion of PD into bronchial secretions.* Three patients with bronchiectasis and markedly purulent sputum were studied. The bronchiectasis was secondary to chronic lymphatic leuke-

mia with mediastinal adenopathy in one patient, to delayed resolution of pneumonia in another and to chronic bronchopneumonia in the third, an 8-year-old child with probable cystic disease of the pancreas. The mean of 7 samples of sputum taken from the three patients before the enzyme was administered was 0.3 units per ml. with a range of 0.1 to 0.4 units per ml. The mean value of 11 samples taken following the intravenous injections of PD was 0.75 units per ml. with a range of 0.1 to 3.2 units per ml. Cytochemical observations on these sputa with the Feulgen stain indicated complete depolymerization of the extracellular DNA in some specimens, inconsistent results in others. It remains to be determined whether the dosage of PD will have to be increased or repeated at more frequent intervals to obtain maximal depolymerization of purulent material in the sputum. Furthermore, respiratory mucin has been shown to exert a protective action on the desoxyribonucleoprotein in the sputum (35) and excessive amounts of mucin may limit the effectiveness of the PD excreted into the bronchial area.

It has been reported that partially purified PD, given for therapeutic purposes by inhalation to patients with chronic bronchiectasis and purulent sputum (35, 36) induced definite depolymerization of the DNA in the sputum with moderate clinical improvement. In 23 cases of uncomplicated purulent bronchitis the administration of 2.5 mgm. of crystalline PD in gelatin buffer by inhalation induced a striking but transitory reduction in the viscosity of purulent sputum, lasting for about 12 hours. When the purulent sputum assumed a mucoid state before the enzyme was given, no change in viscosity followed inhalation of PD. The authors conclude that the volume of sputum, cytochemical observations, bronchospirometer studies, and clinical evaluation of the experimental and control groups failed to reveal a significant difference between treated and untreated groups, probably due to inadequate dosage. They also felt that the protective effect of respiratory mucin may have prevented adequate mixing or contact of the enzyme (PD) and the substrate (desoxyribonucleoprotein) present in the purulent sputum. Consequently, a respiratory mucinase should be used in conjunction with PD in cases where the respiratory mucin is very high in viscosity.

5. *Diffusion into cerebro-spinal canal.* Since the blood-brain barrier is resistant to the passage of large molecules it was of interest to determine whether measurable quantities of PD could be recovered from the cerebro-spinal fluid following the intravenous injection of PD. The content of endogenous DNase in 25 normal cerebro-spinal fluids was found to average 0.05 of a unit per ml.

Intravenous injections of 1,000,000 units of PD were given to 19 patients and the DNase levels determined on the cerebro-spinal fluid from 40 lumbar punctures at varying intervals following the injections (Table II). The PD in the cerebro-spinal fluid of three of six patients with normal meninges, 2 to 6 hours following the injection, varied from 0.1 to 0.4 units per ml. at the fourth or fifth hour, and declined irregularly thereafter to pre-injection levels within the subsequent 24 hours. The average amount of PD recovered from the cerebro-spinal fluid of patients with meningitis was lower than that recovered from the cerebro-spinal fluid of normal individuals. It appears probable that the same type of combination of enzyme and substrate (DNA) occurred in meningitic exudation as in the pleural fluids already discussed.

This assumption was supported by the observation that 5 to 15 units per ml. of PD were found in the cerebro-spinal fluid of *normal* individuals 24 hours after the injection of 50,000 units intrathecally. In patients with meningitis, only 1 to 5 units of PD per ml. were found in the cerebro-spinal fluid 24 hours after intrathecal injection, when the cerebro-spinal fluid white blood cell counts were below 10,000 per mm^3. No PD was found at the 24-hour period in the cerebro-spinal fluid of patients with more than 15,000 white blood cells per mm^3.

Patients with tuberculous meningitis complicated by cerebro-spinal fluid block are being treated by both intravenous and intrathecal injections of PD. In two out of three instances the block was relieved as demonstrated by manometric and X-ray studies, and in one instance by autopsy. When sufficiently comprehensive information has been obtained relative to the problem of block, especially in tuberculous meningitis, the results will be reported in a separate communication.

Antibody formation

Streptodornase has been reported to induce the formation of specific antibodies in patients who received Varidase® (streptokinase-streptodornase) for the treatment of local purulent exudations (*7*). Antibody determinations were performed on sera from 12 normal controls and 7 patients selected at random from those who had received single injections of 500,000 to 1,000,000 units of PD. The samples of sera in the patient group were obtained 7 to 21 days after the injection of PD. Although methods similar to those previously employed (*7*) were used no antibody was found.

In addition, sera were examined from each of the six patients mentioned previously who had received a total of 60,000,000 units of PD. One child had been given about 1,500,000 units of PD in two intravenous injections and 16 intrathecal injections over a period of two months. Serum samples were taken at the end of the first month, second month, fifth month, and seventh month. No antibody was found. All the other patients in this group received a minimum of 5,000,000 units of PD over varying periods of time ranging from 5 to 45 days. Serum samples were obtained from these patients one to four weeks following the last injection. In one instance a titre of 40 antibody units was obtained, and in two other instances titres of about 5 to 10 units were found. The sera from the other three patients in this group contained no antibody. Specificity of the antibody was indicated inasmuch as PD was neutralized by the anti-serum after the sera was heated at 56° C. for as long as 10 hours, thus excluding the action of natural inhibitor. Furthermore, the heat stable antibody was concentrated in the gamma globulin fraction of the serum by ammonium sulfate fractionation. This antibody showed no cross-reactivity with SD (*7*, *37*).

The evidence indicates, therefore, that the crystalline enzyme employed is a relatively poor antigen in man when injected by the intravenous or intrathecal route. The low antigenicity of PD when injected intravenously in rabbits has been reported (*6*). Variations in the quantity of protein required for effective antigenicity are well known. According to determinations in this laboratory the specific activity of PD is about 600 units/γN, or approximately 11 mgm. of crystalline protein/1,000,000 units of PD. For comparative purposes the subcutaneous injection of 0.5 mgm. of purified diphtheria toxoid protein is effective as a primary antigen in man.

Potential therapeutic implications

The rationale of the possible therapeutic usefulness of PD rests upon the results of previous studies of streptococcal desoxyribonuclease (contained in Varidase®), and the similarity of the enzymatic action of PD and streptococcal desoxyribonuclease.

The identification of desoxyribose nucleoprotein (DNA-P) as a significant constituent of purulent exudations which frequently accounts for their thick, viscid physical characteristics (*38*), initiated and broadened the studies of streptococcal enzymes in therapy of which SD is a significant component (*39*, *40*).

It is unnecessary to review again the details of the mechanism by which SD promotes recovery except to point out that the basic features of its therapeutic usefulness are immediately dependent upon the thinning and destructive effect of the enzyme on the viscous DNA-P (*41*, *42*). Previous reports on the effectiveness of SK-SD (Varidase®) in the local treatment of purulent infections (*43–45*) have been amply confirmed. (For recent references see footnote 14 to list of References.)

In considering the two enzymes, PD and SD, the partially purified preparations of SD have been reported to contain several nucleolytic enzymes by which DNA is extensively split with the ultimate liberation of free purine bases (*46*). On the other hand, PD splits DNA only to the level of tetra-nucleotides (*4*, *47*). In both instances, degradation has occurred to the level of acid-soluble, dialyzable fragments.

When, in the present studies, it was demonstrated that PD exhibited the unusual capacity both of renal excretion and diffusion into various extravascular areas following intravenous injection, it became evident that local areas of disease not easily accessible for topical application of the enzyme might be reached *via* the intravenous route.

TABLE II

PD in the cerebro-spinal fluid of twelve patients with normal meninges, and six with meningitis at varying time intervals following the injection of 1,000,000 units intravenously

Time* (hours)	DNase in spinal fluid (units)	Patients with normal meninges (N), or meningitis (M)
0	0.05	Average pre-injection level on all patients N and M. Range 0.0–0.15
2	0.2	N
2	0.1	N
4	0.1	N
5	0.4	N
5½	0.4	N
6	0.1	N
7	0.05	N
7	0.10	M
12	0.0	N
13½	0.1	N
15	0.1	N
24	0.2	M
24	0.0	M
24	0.1	N
24	0.1	N
24	0.0	M
48	0.0	M
48	0.0	M

* Time (hours) indicates time after the intravenous injection when sample of spinal fluid was obtained.

Since some of the patients employed in the present investigation suffered from diseases for which local dornase therapy might be indicated, the fact that PD reached the area in an enzymatically active state and caused depolymerization of the local collections of DNA-P is of considerable interest. The patient with cystitis (Figure 7) is a striking example of the mechanism just outlined. In some of the other patients with either empyema, purulent bronchitis or meningitis, the intravenously injected PD reached the site of disease and exhibited transient local activity. Further studies directed primarily toward the development of the optimal method of utilizing PD therapeutically, particularly by intravenous injections, are now in progress.

SUMMARY

1. The intravenous injection of bovine crystalline pancreatic desoxyribonuclease into man in doses up to 2,000,000 units, evoked no demonstrable toxic reactions either general, local, or of the hematopoietic system.

2. The introduction of the enzyme intrathecally into adults and children with normal meninges, in an average dose of 50,000 units was accompanied by slight changes in the cytology and total protein of the cerebro-spinal fluid. An occasional transient reduction in the cerebro-spinal fluid pressure was also noted. No irritative effect was observed following intrathecal injections of PD into patients with meningitis.

3. The blood level one hour following an intravenous injection of 1,000,000 units averaged about 30 units per ml. of serum and fell to near normal values within 24 hours.

4. The measured level of pancreatic desoxyribonuclease in the serum was found to be partially dependent upon the amount of an inhibitor for PD present in the circulating blood of the patients. The inhibitor proved to be a heat labile, non-dialyzable protein that combined reversibly with PD in a readily dissociable complex.

5. Pancreatic desoxyribonuclease in an active form was excreted in the urine following its injection intravenously in patients. Following rapid injections, about 8 per cent of the intravenous dose was excreted 10 to 12 hours post-injection. The renal excretion of this protein of the albumin type presents certain unusual aspects which have been discussed.

Endogenous desoxyribonuclease was also found to be excreted in the urine of normal individuals.

6. Intravenously injected pancreatic desoxyribonuclease diffused readily into the peritoneal and pleural fluids, wound exudates, and bronchial secretions. A dosage of 1,000,000 units gave a range of diffusion of 0.1 to 3.2 units per ml. The enzyme also diffused into the cerebro-spinal fluid, but in smaller amounts and with some irregularity.

7. In 13 patients serologically studied, no specific antibody was demonstrable in 10; in the remaining 3 patients, only low titres occurred.

8. The potential therapeutic implications associated with the unusual excretion and diffusion of pancreatic desoxyribonuclease are discussed.

ACKNOWLEDGMENT

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REFERENCES [14]

1. Johnson, A. J., and Goger, P. R., The intravenous injection into patients of crystalline pancreatic desoxyribonuclease. Its excretion in the urine and its distribution in certain body fluids. J. Clin. Invest., 1953, **32**, 579 (Abstr.).
2. Kunitz, M., Isolation of crystalline desoxyribonuclease from beef pancreas. Science, 1948, **108**, 19.
3. Kunitz, M., Crystalline desoxyribonuclease. I. Isolation and general properties. Spectrophotometric method for the measurement of desoxyribonuclease activity. J. Gen. Physiol., 1950, **33**, 349.
4. Kunitz, M., Crystalline desoxyribonuclease. II. Digestion of thymus nucleic acid (desoxyribonucleic acid). The kinetics of the reaction. J. Gen. Physiol., 1950, **33**, 363.
5. Christensen, L. R., Methods for measuring the activity of components of the streptococcal fibrinolytic system, and streptococcal desoxyribonuclease. J. Clin. Invest., 1949, **28**, 163.
6. McCarty, M., Purification and properties of desoxyribonuclease isolated from beef pancreas. J. Gen. Physiol., 1946, **29**, 123.
7. Hazlehurst, G. N., Anti(streptococcal) desoxyribonuclease. Occurrence in the sera of patients treated with streptococcal concentrates containing streptodornase. J. Immunol., 1950, **65**, 85.
8. Johnson, A. J., and Tillett, W. S., The lysis in rabbits of intravascular blood clots by the streptococcal fibrinolytic system (streptokinase). J. Exper. Med., 1952, **95**, 449.
9. Sherry, S., Johnson, A., and Tillett, W. S., The action of streptococcal desoxyribose nuclease (streptodornase), *in vitro* and on purulent pleural exudations of patients. J. Clin. Invest., 1949, **28**, 1094.
10. Stefanini, M., Purification of the resin amberlite IR-100 for blood coagulation studies. Proc. Soc. Exper. Biol. & Med., 1948, **67**, 22.
11. Gilbert, L. M., Overend, W. G., and Webb, M., The inhibition of pancreas deoxyribonuclease. Exptl. Cell Research, 1951, **2**, 349.
12. Wintrobe, M. M., Clinical Hematology, 3rd ed., Philadelphia, Lea and Febiger, 1951, pp. 262 and 256.
13. Shapiro, S., Weiner, M., Luddecke, H. F., Kroc, R. L., and White, E. J., A simply prepared, standardized, and relatively stable thromboplastin extract for estimation of prothrombin time. Am. Heart J., 1950, **40**, 766.
14. Mathis, H. B., A simple office procedure for demonstrating lupus erythematosus cells in peripheral blood. Blood, 1951, **6**, 470.
15. Magath, T. B., and Winkle, V., Technic for demonstrating "L.E." (lupus erythematosus) cells in blood. Am. J. Clin. Path., 1952, **22**, 586.
16. Kingsbury, F. B., Clark, C. P., Williams, G., and Post, A. L., The rapid determination of albumin in urine. J. Lab. & Clin. Med., 1926, **11**, 981.
17. Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical Physiological Chemistry, 12th ed., London, J. A. Churchill, Ltd., 1947, p. 520.
18. Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical Physiological Chemistry, 12th ed., London, J. A. Churchill, Ltd., 1947, p. 814.
19. Eagle, H., and Harris, T. N., Studies in blood coagulation: V. The coagulation of blood by proteolytic enzymes (Trypsin, Papain). J. Gen. Physiol., 1937, **20**, 543.
20. Tagnon, H. J., Effect of intravenous injection of trypsin on the blood coagulation time in hemophilia. Proc. Soc. Exper. Biol. & Med., 1944, **57**, 45.
21. Kurnick, N. B., The determination of desoxyribonuclease activity by methyl green; application to serum. Arch. Biochem., 1950, **29**, 41.
22. Klemperer, P., Gueft, B., Lee, S. L., Leuchtenberger, C., and Pollister, A. W., Cytochemical changes of acute lupus erythematosus. Arch. Path., 1950, **49**, 503.
23. Kurnick, N. B., Pariser, S., Schwartz, L. I., Lee, S. L., and Irvine, W., Studies on desoxyribonuclease in systemic lupus erythematosus. Nonparticipation of serum desoxyribonuclease in the "L.E. phenomenon." J. Clin. Invest., 1952, **31**, 1036.
24. Wróblewski, F., and Bodansky, O., Presence of desoxyribonuclease activity in human serum. Proc. Soc. Exper. Biol. & Med., 1950, **74**, 443.
25. Kurnick, N. B., Desoxyribonuclease activity of sera of man and some other species. Arch. Biochem. & Biophys., 1953, **43**, 97.
26. Zamenhoff, S., Personal Communication.
27. Kurnick, N. B., and Carrera, A. E., Reduction in plasma desoxyribonuclease activity on circulation through the liver: index of liver function. Proc. Soc. Exper. Biol. & Med., 1953, **84**, 618.
28. Henstell, H. H., and Freedman, R. I., The viscosimetric determination of desoxyribonuclease inhibition. Cancer Research, 1952, **12**, 341.
29. Henstell, H. H., Freedman, R. I., and Ginsburg, B., An inhibitor of desoxyribonuclease in human white blood and bone marrow cells, and its relationship to cellular maturity. Cancer Research, 1952, **12**, 346.
30. Kurnick, N. B., Schwartz, L., Pariser, S., Lee, S., and Irvine, W., The role of desoxyribonuclease and a nuclease inhibitor from leucocytes in the lupus erythematosus cell phenomenon. J. Clin. Invest., 1952, **31**, 645.
31. Dabrowska, W., Cooper, E. J., and Laskowski, M., A specific inhibitor for desoxyribonuclease. J. Biol. Chem., 1949, **177**, 991.

[14] For comprehensive review of recent articles on clinical use of Varidase® (streptokinase-streptodornase), see bibliography contained in: "Recent advances in Varidase therapy" distributed by Lederle Laboratories Division, American Cyanamid Co., 30 Rockefeller Plaza, New York 20, N. Y.

32. Cooper, E. J., Trautmann, M. L., and Laskowski, M., Occurrence and distribution of an inhibitor for desoxyribonuclease in animal tissues. Proc. Soc. Exper. Biol. & Med., 1950, **73**, 219.

33. Laskowski, M., Personal Communication.

34. Chinard, F. P., Lauson, H. D., Eder, H. A., Greif, R. L., and Hiller, A., A study of the mechanism of proteinuria in patients with the nephrotic syndrome. J. Clin. Invest., 1954, **33**, 621.

35. Elmes, P. C., and White, J. C., Deoxyribonuclease in the treatment of purulent bronchitis. Thorax, 1953, **8**, 295.

36. Armstrong, J. B., and White, J. C., Liquefaction of viscous purulent exudates by deoxyribonuclease. Lancet, 1950, **2**, 739.

37. McCarty, M., The inhibition of streptococcal desoxyribonuclease by rabbit and human antisera. J. Exper. Med., 1949, **90**, 543.

38. Sherry, S., Tillett, W. S., and Christensen, L. R., Presence and significance of desoxyribose nucleoprotein in the purulent pleural exudates of patients. Proc. Soc. Exper. Biol. & Med., 1948, **68**, 179.

39. Tillett, W. S., Sherry, S., and Christensen, L. R., Streptococcal desoxyribonuclease: Significance in lysis of purulent exudates and production by strains of hemolytic streptococci. Proc. Soc. Exper. Biol. & Med., 1948, **68**, 184.

40. McCarty, M., The occurrence of nucleases in culture filtrates of group A hemolytic streptococci. J. Exper. Med., 1948, **88**, 181.

41. Tillett, W. S., and Sherry, S., The effect in patients of streptococcal fibrinolysin (streptokinase) and streptococcal desoxyribonuclease on fibrinous, purulent, and sanguinous pleural exudations. J. Clin. Invest., 1949, **28**, 173.

42. Tillett, W. S., Studies on the enzymatic lysis of fibrin and inflammatory exudates by products of hemolytic streptococci. Harvey Lectures, Series XLV, 1949–50, Charles C Thomas, 1952, p. 149.

43. Tillett, W. S., Sherry, S., and Read, C. T., The use of streptokinase-streptodornase in the treatment of postpneumonic empyema. J. Thoracic Surg., 1951, **21**, 275.

44. Tillett, W. S., Sherry, S., and Read, C. T., The use of streptokinase-streptodornase in the treatment of chronic empyema, with an interpretive discussion of enzymatic actions in the field of intrathoracic diseases. J. Thoracic Surg., 1951, **21**, 325.

45. Sherry, S., and Tillett, W. S., The local use of streptokinase-streptodornase in chronic refractory areas of suppuration with draining sinuses. Ann. Surg., 1952, **135**, 479.

46. Sherry, S., and Goeller, J. P., The extent of the enzymatic degradation of desoxyribonucleic acid (DNA) in purulent exudates by streptodornase. J. Clin. Invest., 1950, **29**, 1588.

47. Carter, C. E., and Greenstein, J. P., Studies on the enzymatic degradation of nucleic acids. J. Nat. Cancer Inst., 1946, **7**, 29.

A STUDY OF THE MODE OF ACTION OF THE ADRENAL MEDULLARY HORMONES ON SODIUM, POTASSIUM AND WATER EXCRETION IN MAN[1, 2]

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The adrenal medullary hormones, *l*-epinephrine and *l*-norepinephrine, elicit differing systemic hemodynamic responses when administered in physiologic dosage to normal man (2, 3). Both produce an elevation in the arterial pressure but *l*-epinephrine does so by increasing cardiac output in association with diminished or unchanging peripheral vascular resistance, whereas *l*-norepinephrine usually induces a fall in cardiac output and an increase in resistance. Both evoke renal vasoconstriction and a reduction in renal blood flow (4–6) with a decrease in the urinary concentration and excretion of sodium and potassium (7, 8). Since glomerular filtration is not affected (5–9) the reduction in electrolyte output may be ascribed to augmented tubular reabsorption. The urine flow remains essentially unchanged or increases during intravenous infusion of either agent and usually declines abruptly upon termination of the pressor response.

These alterations in urine formation may be ascribed to a direct action of the adrenal medullary hormones upon the tubular cellular processes of salt and water transfer or to an indirect effect upon tubular function mediated by release of adrenal cortical hormones or neurogenic activity. The change in urine flow may reflect a secondary change in the production of the antidiuretic hormone (ADH) by the posterior pituitary. The possibility that local renal hemodynamic adjustments also play an important role cannot be excluded. The study reported in this paper was undertaken in an attempt to resolve these uncertainties regarding the mechanism of the renal functional response to *l*-epinephrine and *l*-norepinephrine.

METHODS

Renal clearance methods were used to evaluate renal hemodynamics and to measure the urinary excretion of sodium and potassium during the response to the intravenous infusion of *l*-epinephrine, *l*-norepinephrine, ephedrine, angiotonin, and S-methyl isothiourea sulfate in twenty-two apparently normal human subjects. They ranged in age from 17 to 62 years, were almost equally divided as to sex, and were clinically free from cardiovascular and renal disease. In addition, the renal functional response to *l*-norepinephrine was determined in two patients with Addison's disease, one patient who had had a bilateral adrenalectomy for carcinoma of the prostate, and one patient with diabetes insipidus. All subjects received a regular hospital diet containing about 10 Gm. sodium chloride daily.

These studies were made in the morning with the subjects in the basal state, resting quietly in bed. Each subject had ingested 500 to 1000 ml. of water one to two hours prior to the study. Saline solution was not administered; all infusions were made up in 5 per cent dextrose in water immediately before using in order to avoid the depression in p-aminohippurate extraction noted by Baldwin, Schreiner, Breed, Wesson, and Maxwell (10) when PAH and glucose are incubated together in distilled water for several hours.

The renal clearance of sodium p-aminohippurate (PAH) [6] or Diodrast® was employed in measuring the effective renal plasma flow. Since the hematocrit values did not change appreciably these values may be equated with the renal blood flow. Inulin clearance served as a measure of glomerular filtration rate. These procedures and the analytical methods employed have been described in detail elsewhere (11, 12). All plasma and urine specimens were analyzed for sodium and potassium with an internal standard flame photometer.

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[2] These data have appeared in preliminary form elsewhere (1).

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[6] We are indebted to Dr. W. Boger and the Medical Research Division of Sharp and Dohme, Inc., Philadelphia, Penna. for a generous supply of sodium p-aminohippurate.

The same general plan was followed throughout. Three ten-minute "control" clearance periods were obtained. The blood pressure, measured sphygmomanometrically, and pulse rate were recorded at frequent intervals. Upon completion of the control measurements an intravenous infusion of the pressor agent (diluted in 5 per cent dextrose in water) was started. The concentration of pressor substance in the infusion was 8 μg. per ml. for *l*-norepinephrine,[7] 10 mg. per ml. for S-methyl isothiourea sulfate, 2 units per ml. for angiotonin,[8] and 2 mg. per ml. for ephedrine. An attempt was made to achieve a sustained increment of 25 to 50 mm. Hg in systolic blood pressure. This pressor response was nearly always achieved with the infusion of *l*-norepinephrine regulated at 12 to 15 μg. per minute, S-methyl isothiourea at 15 mg. per minute, angiotonin at 5 units per minute and ephedrine at 5 mg. per minute. When the blood pressure had stabilized for ten to fifteen minutes, the bladder was emptied, washed out with distilled water and the urine discarded. Three ten-minute "pressor" clearance periods were then collected. During the infusion of pressor drug, blood pressure and pulse rate were recorded every two minutes. The infusion of the drug was discontinued and ten to fifteen minutes were allowed for equilibration. The urine obtained during this period was discarded and three ten-minute "recovery" clearance periods were obtained. Again, the blood pressure and pulse rate were measured frequently (at least once each minute).

Two normal subjects were infused with *l*-epinephrine[9] in 5 per cent dextrose (2 μg. per ml). The infusions were maintained at a rate of 2 μg. per minute, an amount insufficient to cause a rise in systemic blood pressure or obvious peripheral circulatory effect.

In four normal subjects high spinal anesthesia (T8 to T4) was induced by the intrathecal instillation of 7.2 mg. of nupercaine. Under the continued influence of high spinal anesthesia, three control renal clearance periods were obtained. The effective renal plasma flow was measured by the clearance of Diodrast®. The renal hemodynamic response and electrolyte and water excretion during administration of *l*-norepinephrine were then observed for two or three additional periods. Only values for those clearance periods obtained when the level of

[7] Supplied through the courtesy of the Medical Research Department of Winthrop Stearns, Inc., New York, as Levophed®.

[8] Generously provided for this study by Dr. Oscar Helmer and the Lilly Laboratory for Clinical Research, Indianapolis, Indiana.

[9] We are indebted to Dr. M. M. Rapport for a supply of pure *l*-epinephrine.

TABLE I

The effect of l-norepinephrine on renal function in adrenal cortical insufficiency *

Subject												
Sex	Age	S.A. M^2	Procedure	B.P. *mm. Hg*	P.R. *min.*	V *ml./min.*	GFR *ml./min.*	RPF *ml./min.*	FF %	$U_{Na}V$ *μEq./min.*	U_KV *μEq./min.*	C_{Na}/C_{in} %	C_K/C_{in} %
Addison's disease													
	R. S.		Control	146/86	74	5.2	110	450	24	406.0	57.7	2.7	13.6
			Infusion	184/120	62	5.7	100	305	33	72.6	34.5	0.5	8.8
F	38	1.84	Recovery	138/90	76	2.2	105	425	25	113.9	26.9	0.8	6.6
	S. G.		Control	112/76	68	1.7	95	360	26	254.6	52.6	2.1	14.5
			Infusion	168/110	56	2.5	90	260	34	63.7	45.0	0.5	13.0
M	43	1.70	Recovery	114/72	80	1.4	75	335	23	141.6	24.1	1.4	8.3
After bilateral adrenalectomy													
	J. J.		Control	126/76	80	3.3	90	510	18	146.6	56.7	1.2	12.7
			Infusion	170/90	64	3.0	85	295	22	57.3	33.0	0.5	7.8
M	58	1.68	Recovery	126/74	80	5.6	90	455	20	115.5	24.7	0.9	5.5

* All values are averages of three determinations. Infusions of *l*-norepinephrine were administered throughout the "infusion" period at a rate sufficient to produce the desired pressor response. The arterial blood pressure (B.P.) was measured sphygmomanometrically. Abbreviations are as follows:

S.A. = body surface (M^2)
P.R. = pulse rate
V = urine flow (ml./min.)
GFR = glomerular filtration rate, inulin clearance (ml./min.)
RPF = renal plasma flow, sodium p-aminohippurate clearance (ml./min.)
FF = filtration fraction—GFR/RPF (%)
$U_{Na}V$ = urinary sodium output (μEq./min.)
U_KV = urinary potassium output (μEq./min.)
C_{Na}/C_{in} = sodium-inulin clearance ratio or proportion of filtered sodium excreted (%)
C_K/C_{in} = potassium-inulin clearance ratio or proportion of filtered potassium excreted (%).

TABLE II

Effect of l-norepinephrine on renal function in a patient with diabetes insipidus and in normal subjects during Pitressin® induced antidiuresis *

Subject Sex	Age	S.A. M^2	Procedure	B.P. mm. Hg	P.R. min.	V ml./min.	GFR ml./min.	RPF ml./min.	FF %	$U_{Na}V$ μEq./min.	U_KV μEq./min.	C_{Na}/C_{in} %	C_K/C_{in} %
Diabetes insipidus (*l*-norepinephrine)													
	D. A.		Control	126/78	70	12.3	150	585	26	218.6	68.5	1.1	10.3
			Infusion	154/90	66	8.7	125	430	29	52.6	57.3	0.3	10.4
F	35	1.78	Recovery	114/72	74	12.5	155	600	26	132.9	33.8	0.6	4.8
Normal subjects during Pitressin® antidiuresis (*l*-norepinephrine)													
	P. M.		Control	116/74	64	12.3	150	690	22	31.6	42.8	0.2	6.8
			Pitressin®	116/76	64	0.8	155	630	25	31.9	44.3	0.2	6.8
			Pit.+*l*-nor.	144/90	60	0.8	155	485	32	24.1	36.8	0.1	5.4
M	17	1.72	Recovery	130/76	60	0.4	160	530	30	25.2	36.7	0.1	5.4
	J. M.		Control	104/64	84	5.0	105	475	22	46.3	13.5	0.3	3.8
			Pitressin®	108/62	82	1.8	100	500	20	43.1	14.7	0.3	6.3
F	38	1.37	Pit.+*l*-nor.	152/92	68	0.8	105	460	24	22.6	8.5	0.3	4.7
	R. S.		Control	112/72	92	6.6	95	490	20	51.3	34.6	0.4	8.9
			Pitressin®	104/74	84	0.9	95	515	24	65.2	34.6	0.4	6.4
			Pit.+*l*-nor.	152/100	56	0.7	90	310	30	22.2	25.0	0.2	6.8
F	24	1.57	Recovery	110/72	80	0.7	140	610	24	51.4	29.3	0.3	5.3
	J. R.		Control	120/74	68	5.5	140	590	24	304.0	102.6	1.6	16.3
			Pitressin®	122/80	68	1.4	145	525	27	304.8	83.6	1.5	11.3
			Pit.+*l*-nor.	166/102	54	1.3	130	400	32	197.5	39.3	1.1	6.8
M	32	1.92	Recovery	126/76	74	1.5	180	650	25	440.5	48.0	2.1	7.0
Normal subjects (*l*-norepinephrine + Pitressin®)													
	K. N.		Control	122/62	96	13.3	95	490	20	78.0	47.0	0.6	12.4
			l-norepi.	146/82	66	7.0	85	295	29	29.1	28.6	0.3	8.4
			l-nor.+pit.	148/86	62	0.6	80	355	23	17.3	16.2	0.2	5.0
F	30	1.46	Recovery	110/62	92	0.4	120	755	16	74.2	31.3	0.5	6.6
	E. B.		Control	102/66	82	7.8	120	500	24	45.9	33.5	0.3	5.8
			l-norepi.	138/92	66	8.7	135	490	27	83.1	27.1	0.5	4.2
			l-nor.+pit.	144/90	62	0.5	105	515	20	85.6	23.3	0.6	4.6
F	25	1.71	Recovery	100/68	80	0.4	135	720	19	48.8	27.4	0.3	4.2
	F. E.		Control	112/74	86	5.7	120	540	20	107.1	69.4	0.6	14.4
			l-norepi.	146/88	78	6.0	105	470	23	92.8	33.5	0.6	7.5
			l-nor.+pit.	150/86	78	0.4	115	445	26	66.2	27.4	0.5	6.0
F	34	1.92	Recovery	106/68	92	0.6	125	580	22	75.7	23.9	0.4	4.8

* All values are for the last of the determinations during the control periods, both infusion periods and the recovery periods, with the exception of the patient with diabetes insipidus (D. A.) in which the values are the usual averages of three determinations in each phase.

anesthesia was at T8 or higher have been included in this study.

Pitressin® (1 milliunit per kg. per hour) was administered intravenously to seven normal subjects. Following completion of the usual control periods in four subjects, the infusion of Pitressin® was started and three additional clearance periods were obtained. An infusion of *l*-norepinephrine was then given in addition for the next three periods. In three subjects after the usual control periods this procedure was reversed; *i.e.*, the effect of *l*-norepinephrine alone was followed for three periods and then during the action of Pitressin® for three additional clearance periods. In all seven subjects recovery was observed in the usual manner upon the termination of the infusions.

RESULTS

The findings are set out in Tables I to V and in the figures. All values appearing in the Tables are averages of three successive determinations except where noted. In Figures 4 to 6, the values for electrolyte and water excretion are expressed as percentages of the averaged control figures in

TABLE III

The effect of l-norepinephrine on renal function in normal subjects during high spinal anesthesia *

Subject Sex	Age	S.A. M^2	Procedure	B.P. *mm. Hg*	P.R. *min.*	V *ml./min.*	GFR *ml./min.*	RPF *ml./min.*	FF %	$U_{Na}V$ *μEq./min.*	U_KV *μEq./min.*	C_{Na}/C_{in} %	C_K/C_{in} %
Normal subjects during high spinal anesthesia (*l*-norepinephrine)													
J. G.			Sp. anes. (T4)	102/68	72	0.5	85	460	19	55.7	55.6	0.5	17.5
M	31	1.60	Sp.+*l*-nor.	132/90	48	0.4	85	360	23	21.0	45.4	0.2	15.5
A. L.			Sp. anes. (T7)	108/60	88	0.9	115	540	23	135.4	61.1	0.8	11.4
M	62	1.96	Sp.+*l*-nor.	138/82	60	0.4	100	400	25	34.1	38.9	0.3	9.5
W. N.			Sp. anes. (T7)	112/62	86	0.6	105	610	17	47.5	28.6	0.3	6.8
M	31	2.03	Sp.+*l*-nor.	128/82	58	0.7	110	540	21	19.9	32.9	0.1	7.3
C. I.			Sp. anes. (T8)*	114/72	72	0.3	95	345	27	16.9	23.4	0.1	7.7
M	43	1.78	Sp.+*l*-nor.*	140/90	58	0.6	95	335	28	19.5	38.1	0.2	12.5

* All values are averages of three determinations except in instances denoted with an asterisk. Control clearance periods were obtained during spinal anesthesia. An infusion of *l*-norepinephrine was administered during the continued influence of the spinal anesthesia. The lowest thoracic level of spinal anesthesia during the entire procedure is noted, and no values were accepted when anesthesia level was lower than T8. Abbreviations are the same as in Table I.

order to make possible comparison of the changes produced by the experimental procedure and to minimize for this purpose the variation between individuals attributable to differences in dietary preparation, appetite, and other uncontrolled variables. Plasma sodium and potassium concentrations were not significantly altered in any of these studies and are not included in the tables.

In most instances, *l*-norepinephrine was used in preference to *l*-epinephrine, because the unpredictable side-effects and variable response to *l*-epinephrine make it much less safe to use than *l*-norepinephrine. However, both agents produce the same changes in renal functions and it may be inferred that the findings reported apply to the adrenal medullary hormones in general.

Changes observed in renal hemodynamics and in the urinary excretion of sodium, potassium and water in response to the adrenal medullary hormones

1. During adrenal cortical insufficiency: The response to intravenous *l*-norepinephrine was observed in two patients with Addison's disease. One (R. S.) was receiving DOCA (pellets, 450 mg., implanted 6 months previously) and the other (S. G.) was maintained on cortisone (12.5 mg. daily—last dose 15 hours prior to study) and DOCA (pellets, 300 mg., implanted 2 weeks previously). In both patients, the renal response to *l*-norepinephrine was typical of that reported in normal subjects (7). The averaged figures for

TABLE IV

The effect of minimal amounts of l-epinephrine on renal function in normal subjects *

Subject Sex	Age	S.A. M^2	Procedure	B.P. *mm. Hg*	P.R. *min.*	V *ml./min.*	GFR *ml./min.*	RPF *ml./min.*	FF %	$U_{Na}V$ *μEq./min.*	U_KV *μEq./min.*	C_{Na}/C_{in} %	C_K/C_{in} %
Normal subjects (minimal amounts of *l*-epinephrine)													
A. H.			Control	102/68	68	5.6	150	780	20	84.7	102.0	0.4	15.1
			Infusion	108/66	68	9.5	150	640	24	58.1	62.6	0.3	9.3
M	33	1.90	Recovery	108/66	68	4.2	150	700	21	59.1	35.0	0.3	5.3
M. K.			Control	112/72	76	6.9	95	625	15	134.8	54.1	1.0	14.8
			Infusion	114/70	76	5.7	100	450	22	92.9	32.1	0.7	8.3
F	44	1.76	Recovery	114/70	76	1.8	110	505	22	110.0	25.7	0.7	6.1

* All values are averages of three determinations. Peripheral circulatory change was avoided by infusion of *l*-epinephrine in an amount insufficient to cause pressor response or change in pulse rate. Abbreviations as in Table I.

TABLE V

The effects of angiotonin, S-methyl isothiourea sulfate and ephedrine on renal function and urinary electrolyte excretion in normal human subjects *

Subject													
Sex	Age	S.A. M^2	Procedure	B.P. *mm. Hg*	P.R. *min.*	V *ml./min.*	GFR *ml./min.*	RPF *ml./min.*	FF %	$U_{Na}V$ *μEq./min.*	U_KV *μEq./min.*	C_{Na}/C_{in} %	C_K/C_{in} %
						(Angiotonin)							
	F. Z.		Control	136/78	72	9.2	145	665	22	485.0	78.6	2.3	13.3
			Infusion	152/94	72	1.7	120	400	31	122.9	64.1	0.7	13.0
M	48	1.96	Recovery	128/80	72	4.4	130	590	22	195.4	86.1	1.0	16.3
	E. H.		Control	138/84	64	7.3	105	495	21	248.7	82.9	1.7	14.7
			Infusion	170/106	58	1.1	65	235	27	50.1	28.3	0.7	9.2
M	44	1.92	Recovery	132/82	68	2.7	95	460	21	135.9	78.4	1.1	15.8
	B. E.		Control	108/70	84	4.9	115	600	19	127.4	53.1	0.8	10.2
			Infusion	150/86	72	1.4	95	365	26	23.2	30.3	0.2	7.3
F	29	1.43	Recovery	104/70	84	6.9	135	640	21	94.9	42.4	0.5	6.9
						(S-methyl isothiourea)							
	P. C.		Control	128/72	64	4.5	130	695	19	74.5	51.4	0.4	10.1
M	24	1.65	Infusion	140/86	50	3.7	95	435	22	29.5	36.0	0.2	9.3
	G. M.		Control	108/70	76	1.6	80	340	24	56.6	21.9	0.5	6.4
			Infusion	130/92	56	0.7	40	140	30	14.7	10.5	0.3	6.2
F	28	1.35	Recovery	126/88	60	0.5	40	160	25	20.7	9.7	0.4	6.0
						(Ephedrine)							
	M. T.		Control	102/58	76	4.9	125	595	21	51.2	61.4	0.3	10.7
			Infusion	128/64	88	4.5	135	585	23	113.7	53.6	0.6	8.8
F	28	1.66	Recovery	132/60	86	1.3	130	585	23	91.4	24.4	0.5	4.2
	E. P.		Control	142/84	80	4.9	125	520	23	249.3	67.7	1.4	11.8
			Infusion	176/90	86	4.6	125	590	21	253.2	62.5	1.4	10.9
M	29	1.96	Recovery	170/88	88	6.3	135	650	20	285.9	58.0	1.6	9.8
	A. G.		Control	116/72	60	10.3	75	335	22	182.2	39.2	1.7	11.0
			Infusion	144/72	70	9.3	80	350	23	291.3	34.1	2.6	9.0
F	55	1.66	Recovery	136/68	70	6.1	85	345	24	237.7	23.9	2.0	6.0
	R. W.		Control	120/78	64	3.4	120	555	21	130.5	98.1	0.9	20.7
			Infusion	164/86	76	8.9	110	590	19	202.7	86.5	1.4	19.1
M	17	1.83	Recovery	156/84	80	3.0	115	565	20	243.0	42.9	1.5	9.5

* All values are averages of three determinations.
Abbreviations are the same as in Table I.

the effective renal plasma flow (RPF), glomerular filtration rate (GFR), urinary sodium excretion ($U_{Na}V$), and urinary potassium excretion (U_KV) during the control periods, during the *l*-norepinephrine infusion, and during the recovery periods are presented in Table I. Renal plasma flow (RPF) decreased during the infusion of norepinephrine. The urinary excretion of sodium ($U_{Na}V$) and potassium (U_KV) declined sharply while glomerular filtration rate (GFR) did not appear to change. Urine volume (V) tended to increase during the pressor period and fell abruptly when *l*-norepinephrine was discontinued. During the recovery periods the effective renal plasma flow returned essentially to control values and urinary sodium output tended to increase, but potassium excretion continued to fall. The changes in systemic and renal hemodynamics, urinary sodium, and potassium excretion and urine volume in one (S. G.) of these patients with Addison's disease are shown in Figures 1 and 2.

One patient (J. J.) with carcinoma of the prostate, who had undergone a total bilateral adrenalectomy (maintained on cortisone—35 mg. daily—last dose 12 hours prior to study) was infused with *l*-norepinephrine in the usual manner. The

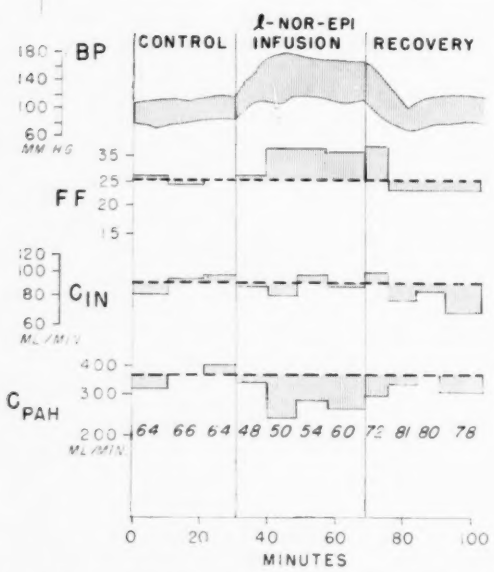

FIG. 1. THE EFFECT OF *L*-NOREPINEPHRINE ON FILTRATION RATE AND RENAL PLASMA FLOW IN A PATIENT WITH ADDISON'S DISEASE (SUBJECT S. G.)

Blood pressure (BP—sphygmomanometer), glomerular filtration rate (C_{in}—inulin clearance), renal plasma flow (C_{pah}—p-amino-hippurate clearance), filtration fraction (FF—$C_{in}/C_{pah} \times 100$) and pulse rate (numerical values inserted below C_{pah}) were measured before, during, and after intravenous infusion of *l*-norepinephrine. All values except those for blood pressure and pulse rate are plotted semi-logarithmically against time. Renal plasma flow and filtration fraction rose with the elevation in blood pressure during the infusion and returned to the control levels during recovery. The values just after beginning and stopping the infusion of pressor agent have been omitted in computing the averages for this patient (S. G.) in Table I because intra-renal delay and the process of equilibration resulted in unreliable clearances.

decrements in renal plasma flow and urinary excretion of sodium and potassium (Table I) were similar to those reported in normal subjects (7), and to those observed in patients with Addison's disease maintained on adequate substitution therapy. The renal plasma flow promptly returned toward control values when the *l*-norepinephrine infusion was stopped. Sodium excretion in the urine tended to increase but urinary potassium excretion continued to decrease during the recovery periods. The change in water excretion was atypical since urine flow did not fall upon termination of the drug infusion, but tended to rise.

2. During anti-diuretic hormone deficiency or excess: When *l*-norepinephrine was administered by intravenous infusion to one patient with well-established and untreated diabetes insipidus, the blood pressure increased and the renal plasma flow diminished without a statistically significant change in filtration rate (Table II). Urinary concentration and excretion of sodium and potassium also decreased during the pressor response as in normal persons, but urine flow fell by 20 per cent on the average. During recovery, the renal plasma flow promptly increased, sodium output

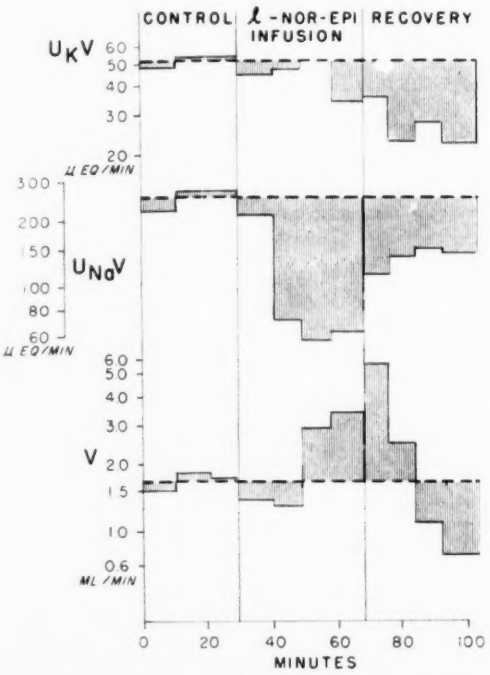

FIG. 2. THE EFFECT OF *L*-NOREPINEPHRINE ON ELECTROLYTE AND WATER EXCRETION IN A PATIENT WITH ADDISON'S DISEASE (SUBJECT S. G.)

Values for potassium (U_KV), sodium ($U_{Na}V$), and water excretion (V) during the study depicted in Figure 1 are plotted semi-logarithmically against time. Electrolyte excretion diminished while urine flow increased. The fall in potassium excretion was delayed and persisted into the recovery period, whereas the changes in sodium and water output tended to return to control values.

rose moderately, but potassium excretion fell still more.

Pitressin® (1 milliunit per kg. per hour) had no statistically significant effect upon blood pressure, pulse rate, renal plasma flow or glomerular filtration rate in four normal subjects. Since determinations made during the period of equilibration were not discarded in these studies, only the last values obtained in successive periods during each phase (control, Pitressin®, or *l*-norepinephrine infusion, Pitressin® and *l*-norepinephrine infusion, and recovery) are set out in Table II in order to provide for a closer approach to the "steady" state. The urine flow always decreased markedly without much change in electrolyte excretion, as others (13) have found. However, sodium output increased by 13 per cent in one subject (R. S.) and potassium excretion decreased by 18 per cent in another (J. R.). When *l*-norepinephrine was administered intravenously after the antidiuretic effect of Pitressin® was well-established and maintained, no change in urine flow occurred and anti-diuresis persisted throughout recovery. The renal plasma flow fell and electrolyte output decreased without change in filtration in every instance. Thus, with the exception of antidiuresis, Pitressin® did not alter the response to *l*-norepinephrine in any significant manner (Figure 3). Likewise, *l*-norepinephrine did not affect the response to Pitressin® in three subjects in whom pressor and renal functional effects of *l*-norepinephrine were established prior to administration of Pitressin® (Table II). In one of these, E. B. (Table II), sodium output increased in association with more or less unchanging renal plasma flow under the influence of *l*-norepinephrine. This anomalous response persisted during the anti-diuretic action of Pitressin®. During recovery after both drugs were withdrawn the urine flow remained depressed and all other values tended to return to control levels. The potassium output continued to fall in F. E. (Table II), however.

3. During high spinal anesthesia: Spinal anesthesia, with loss of sensation up to T8 or higher, caused no statistically significant fall in arterial blood pressure in four normal subjects. Renal plasma flow and filtration rate were unaffected, as others (14) have reported. When *l*-norepinephrine was infused, the usual decrease in renal

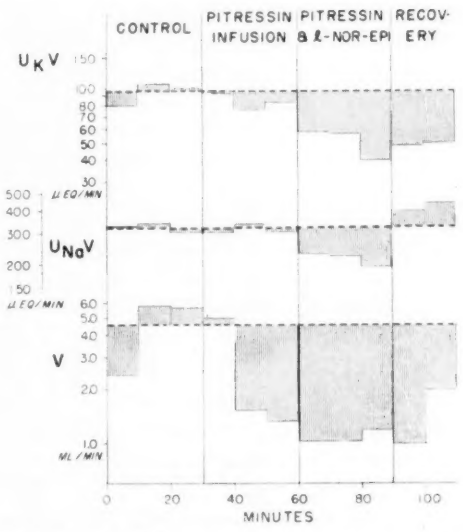

FIG. 3. THE EFFECT OF *L*-NOREPINEPHRINE ON ELECTROLYTE AND WATER EXCRETION DURING PITRESSIN® ANTIDIURESIS (SUBJECT J. R.)

Infusion of Pitressin® produced no significant change in potassium ($U_K V$) and sodium ($U_{Na} V$) excretion, but urine flow (V) fell markedly. During *l*-norepinephrine infusion both potassium and sodium output decreased without change in urine flow.

plasma flow (Diodrast® clearance) (Table III) occurred in all but one (C. I.) while glomerular filtration rate remained unchanged in all. *L*-norepinephrine caused no consistent change in urine flow. The urinary excretion of sodium decreased in all but one subject (C. I.), who presented unusually low values for sodium output during the control periods. Urinary potassium excretion decreased in two (J. G. and A. L.), increased in one (C. I.), and did not change significantly in the other subject (W. N.). It is noteworthy that in C. I., *l*-norepinephrine failed to reduce sodium and potassium output in association with a failure to diminish renal plasma flow. In general, however, high spinal anesthesia did not interfere with the usual changes in renal functional responses to *l*-norepinephrine.

4. In the absence of a pressor response to l-epinephrine: When *l*-epinephrine [10] was administered

[10] *L*-epinephrine was chosen for this study, because *l*-norepinephrine, even in minute dosage, caused at least some degree of peripheral vasoconstriction and arterial hypertension.

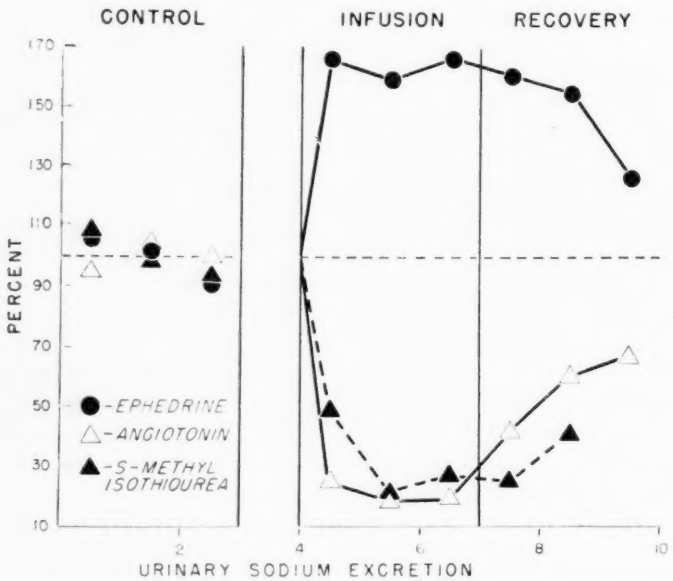

FIG. 4. THE EFFECT OF EPHEDRINE, ANGIOTONIN, AND S-METHYL ISOTHIOUREA SULFATE ON URINE FLOW

The values for urine flow during each "period" (usually 10 to 15 minutes in duration) have been averaged and expressed here as the percentage of the pooled and averaged control figures. An infusion of ephedrine (closed circles), angiotonin (open triangles), or S-methyl isothiourea sulfate was administered intravenously during the periods designated "Infusion." In each study values were obtained during three periods in phase (control, infusion, and recovery); urine collected during the period immediately after starting the infusion was discarded. See Table V for further information on the studies collated here. The urine flow was usually rising during the control periods owing to preliminary ingestion of water.

In subjects receiving ephedrine the peak of the diuretic response was reached shortly after the infusion of ephedrine was started; the urine flow decreased sharply thereafter and stabilized at values at approximately 70 per cent of control on the average. With infusion of angiotonin and S-methyl isothiourea sulfate, urine flow fell abruptly, tending to return to control values on recovery from angiotonin and remaining depressed after S-methyl isothiourea.

intravenously at a rate of 2 μg. per minute to two normal subjects, neither arterial blood pressure nor pulse rate changed significantly and skin pallor did not develop.

Despite the apparent lack of peripheral circulatory adjustment during the *l*-epinephrine infusion, effective renal plasma flow decreased and the urinary excretion of both sodium and potassium fell sharply (Table IV). Glomerular filtration rate did not change significantly. In one subject (A. H.) urine flow increased strikingly during the drug infusion and remained relatively unchanged in the other subject (M. K.). When *l*-epinephrine was discontinued the urine flow in both subjects fell abruptly. During recovery the urinary sodium excretion tended to return toward control values in both subjects, but urinary potassium excretion continued to decrease despite the return of renal plasma flow toward control levels. These changes did not differ appreciably from those observed in response to *l*-epinephrine or *l*-norepinephrine when arterial pressure increased (7).

Comparison of changes in renal hemodynamics and in urinary excretion of water, sodium, and potassium during the action of other pressor agents

1. Angiotonin and S-methyl isothiourea: In order to assess the role of renal hemodynamic adjustments in producing the change in urine formation during the action of the adrenal medullary hormones, the response to other agents having a similar action was studied. For this purpose, angiotonin and S-methyl isothiourea sulfate were chosen, because it is known (15, 16) that both induce renal vasoconstriction and systemic hemodynamic alterations similar to those produced by *l*-norepinephrine. Neither substance is chemically similar to the medullary hormones, and they would seem the less likely, therefore, to behave as analogues in bringing about changes through direct action upon the tubular cells.

Angiotonin (5 units per minute) was administered intravenously to three, and S-methyl isothiourea sulfate (15 mg. per minute) to two normal individuals. Both agents raised the arterial pressure and reduced the pulse rate in association with renal vasoconstriction. In every instance renal plasma flow and glomerular filtration rate decreased (Table V) with a rise in the filtration fraction.

The urine flow always decreased, returning to or toward control levels during recovery from the action of angiotonin and remaining depressed following S-methyl isothiourea sulfate (Figure 4). The decrement in water excretion was relatively greater than the fall in filtration and electrolyte excretion during the infusion of angiotonin, so that the urinary concentration of electrolyte and inulin increased, indicating a relative increment in water reabsorption. S-methyl isothiourea sulfate, on the other hand, depressed urine flow no more than filtration and much less than electrolyte excretion.

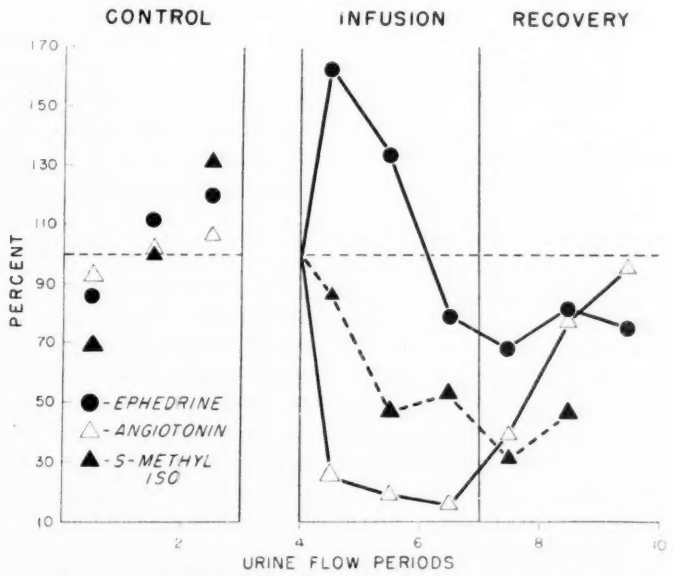

FIG. 5. THE EFFECT OF EPHEDRINE, ANGIOTONIN, AND S-METHYL ISOTHIOUREA SULFATE ON URINARY SODIUM EXCRETION

The data for urinary sodium excretion in the subjects presented in Figure 4 are treated here in a similar fashion. Sodium excretion decreased markedly during infusion of angiotonin and S-methyl isothiourea sulfate, and increased by 70 per cent on the average during ephedrine infusion. There was a tendency to return toward the control values during recovery in all instances.

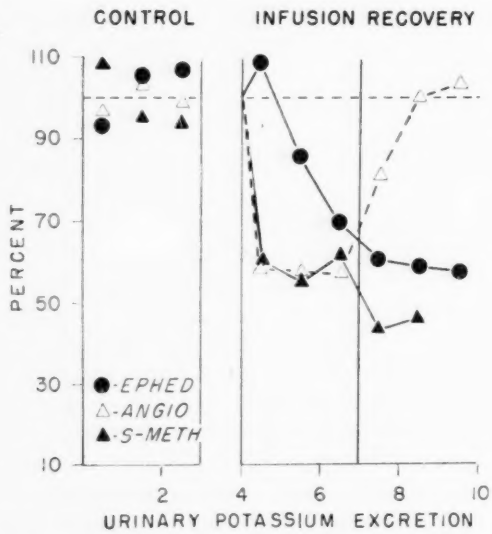

FIG. 6. THE EFFECT OF EPHEDRINE, ANGIOTONIN, AND S-METHYL ISOTHIOUREA SULFATE ON URINARY POTASSIUM EXCRETION

The data are handled as in Figures 4 and 5. Potassium excretion declined during the pressor action of all three agents, returned to the control values after angiotonin and remained depressed during recovery after ephedrine and S-methyl isothiourea.

Sodium and potassium output (Figures 5 and 6) fell off markedly during the action of both drugs. Sodium clearance decreased more than filtration, *i.e.*, the sodium-inulin clearance ratio fell (Table V), whereas potassium clearance tended to decrease to relatively the same extent as, or somewhat more than filtration. All values tended to rise during the recovery period.

These changes indicate a relative increase in tubular reabsorption of electrolytes, but owing to the fact that filtration usually decreased greatly, the actual quantities of sodium, potassium, and water reabsorbed by the tubules diminished.

2. Ephedrine: The effect of ephedrine upon urine function was investigated in four normal persons (Table V) in order to determine whether a substance chemically analogous to *l*-epinephrine would produce similar changes in function. Ephedrine is known to affect the cardiovascular system in more or less the same manner as *l*-epinephrine, but its effect on renal hemodynamics is uncertain. Ranges and Bradley (5) observed renal ischemia in normal human subjects following administration of single doses of ephedrine (45 to 50 mg. subcutaneously). In subsequent studies Maxwell, Morales, and Crowder (17) were unable to detect any change in renal plasma flow following similar doses of the drugs. Their findings have been confirmed in the course of the studies reported here (Table V). Neither renal plasma flow nor glomerular filtration changed, except to increase slightly, in any subject during infusion of ephedrine (2 mg. per min.). Since the arterial pressure rose, the renal vascular resistance must have increased proportionately.

The changes in urine formation during the pressor action of ephedrine differed from those produced by *l*-epinephrine and *l*-norepinephrine. The averaged urine flow did not change in three subjects, but rose markedly in one. During the "recovery" period, urine flow decreased in two, rose in one, and returned to the control value in one study (Table V, Figure 4). Sodium output always increased, in two studies remaining elevated and in two decreasing during recovery. Since filtration did not change, the sodium-inulin clearance ratio rose, indicating decreased tubular reabsorption (Table V, Figure 5). The tubular reabsorption of potassium appeared to increase, since potassium output and the potassium-inulin clearance ratio decreased (Table V, Figure 6).

DISCUSSION

The extent, rapidity, and variety of the physiologic changes observed during intravenous infusion of *l*-epinephrine and *l*-norepinephrine make analysis of their mode of action exceedingly difficult. Both agents probably act directly upon tissues throughout the body. Nonetheless, indirect secondary effects may play a more prominent role in determining the total physiologic response. This consideration is particularly important in an evaluation of the changes in kidney function. It seems reasonable to attribute intra-renal vasoconstriction to direct stimulation of the arteriolar musculature by the circulating hormone, but the changes in urine formation could be ascribed to secondary release of other humoral agents, to neurogenic activity, or to local hemodynamic adjustments.

The results of this study indicate clearly that the retention of sodium and potassium during the pressor action of *l*-norepinephrine (and presumably, also, of *l*-epinephrine) does not require the active intervention of the adrenal cortex. The typical response was observed in patients with Addison's disease or after bilateral adrenalectomy. Although these patients were maintained on adequate substitution therapy, the pathology or absence of the adrenals precluded active adrenocortical participation in the response to norepinephrine.

A change in the production of antidiuretic hormone by the posterior pituitary may account for the alteration in water excretion. Tubular reabsorption of electrolytes is believed to be iso-osmotic (18) and the increased electrolyte reabsorption induced by *l*-norepinephrine should have entailed a corresponding increment in water reabsorption. However, urine flow remained unchanged or actually increased. Although the additional fluid administered during the infusion of *l*-norepinephrine may have contributed, it is likely that suppression of ADH secretion by the action of the medullary hormone played an important role under these circumstances (19). Thus, urine flow decreased in association with the decrement in electrolyte excretion in a patient with diabetes insipidus (Table II). It may be inferred that inability to suppress further the elaboration of ADH "unmasked" the potentially anti-diuretic effect of increased tubular electrolyte reabsorption. In contrast, in a patient with Addison's disease who drank a liter of water without a diuretic response just prior to the study illustrated in Figures 1 and 2, the urine flow increased only during the infusion of *l*-norepinephrine. Since *l*-norepinephrine did not block the antidiuretic response to Pitressin®, this phenomenon suggests that a reduction in ADH formation corrected temporarily the impaired diuretic response of Addison's disease (20). The oliguria usually observed following withdrawal of *l*-norepinephrine or *l*-epinephrine could be accounted for by excessive release of ADH following the period of suppression. *L*-norepinephrine elicited a fall in sodium and potassium output without change in urine flow during antidiuresis imposed by preliminary administration of Pitressin®, strongly suggesting that the changes in electrolyte and water reabsorption occur independently.

The kidney receives a rich nerve supply from the autonomic nervous system through which the changes in tubule function and the renal circulation could have been mediated (18). Spinal anesthesia to levels sufficiently high to interrupt the neural outflow to the kidney did not affect the response to the usual doses of *l*-norepinephrine.[11] Although it is possible that denervation was not complete under these circumstances, even a partial effect should have interfered with the response to some extent if it were neurogenically mediated. Despite successful maintenance of the arterial pressure more or less unchanged, all these patients were incapable of appropriate adjustment to postural change so that the blood pressure tended to fall, indicating widespread loss of splanchnic autonomic activity in which the kidney probably participated. Activation of an intra-renal neural network by the direct action of *l*-norepinephrine circulating in the blood cannot be excluded.

Distention of the renal vasculature and stimulation of local stretch receptors by the elevation in arterial pressure might have also set in train local readjustments. However, *l*-epinephrine produced the usual alterations in electrolyte excretion even when administered in doses too small to change the blood pressure. When hypertension developed during the infusion of angiotonin or S-methyl isothiourea the renal functional response did not altogether resemble that produced by the medullary hormones. Sodium and potassium output diminished but the change could have been attributed to the concomitant fall in glomerular filtration. Reabsorption of sodium and potassium did increase relative to filtration but total reabsorption diminished. The pressor response to ephedrine was associated with an even more marked divergence in the pattern of renal functional response. Neither renal blood flow nor glomerular filtration changed significantly, and the output of sodium actually increased. It may be concluded, therefore, that the local pressor response *per se* was of little or no importance in determining the alteration in renal function.

Renal ischemia, on the other hand, appeared to be invariably associated with diminished sodium output. Angiotonin, S-methyl isothiourea sulfate,

[11] All these patients were oliguric during the control periods presumably owing to release of antidiuretic hormone.

l-epinephrine, and *l*-norepinephrine all induced vasoconstrictive renal ischemia and a reduction in sodium excretion. When ischemia did not develop despite intra-renal vasoconstriction (as in subjects E. B. and C. I. [Tables II and III] during administration of *l*-norepinephrine) or during the action of ephedrine, sodium excretion remained unchanged or increased. These facts suggest that reduction of the blood flow to tubular tissue may affect sodium reabsorptive activity. Pullman and McClure (21) failed to find any significant change in sodium excretion in six normal young men during infusion of *l*-norepinephrine, despite marked reduction in renal blood flow. It is possible that administration of isotonic saline, the smaller doses of the drug employed throughout the better part of each experiment, and the short period of study at dosage levels comparable to those reported here may account for the absence of a change in sodium output. The specific changes that modify potassium excretion remain obscure. The output of potassium was reduced, not only during the pressor action of each drug, but also when blood pressure did not change. The only common factor in these diverse responses appeared to be intra-renal vasoconstriction.

It is quite possible that the adrenal medullary hormones act directly upon renal tubular cells to accelerate electrolyte reabsorption. These substances are highly active and are known (22) to affect various cell types and various intra-cellular activities. The fact that *l*-norepinephrine failed to reduce sodium excretion when the renal blood flow did not decrease is opposed to this possibility. The plasma concentration of norepinephrine may have been too low in these instances but the blood pressure did rise. The response to ephedrine was unexpected and at variance with the hypothesis (23) that it acts only through the intermediation of epinephrine by blocking amine oxidase, thus preventing oxidation of epinephrine. It is possible that the effect of ephedrine on sodium excretion is attributable to one of its metabolites, to insufficient local release of epinephrine, or, as the data suggest, to its failure to induce a reduction in renal blood flow.

SUMMARY

This study has confirmed previous observations of diminished sodium and potassium excretion and a tendency to loss of water in the urine during the pressor action of the adrenal medullary hormones in man.

The change in electrolyte excretion is apparently not mediated through adrenal cortical activation. Three subjects with adrenal cortical insufficiency (two with Addison's disease and one after bilateral adrenalectomy) responded to *l*-norepinephrine like normal persons. Diminished formation of antidiuretic hormone may account for sustained or augmented output of a dilute urine during infusion of the medullary amines. In a patient with diabetes insipidus, *l*-norepinephrine depressed both electrolyte and water excretion. Pitressin® evoked a marked reduction in urine flow without changing electrolyte excretion in three normal subjects receiving an infusion of *l*-norepinephrine. In four subjects administration of *l*-norepinephrine during antidiuresis produced by an infusion of Pitressin® resulted in diminished electrolyte excretion without a change in urine flow, indicating the independence of the changes in electrolyte and water reabsorption by the renal tubules.

Since high spinal anesthesia (T8 or higher) did not interfere with the action of *l*-norepinephrine in four subjects, the response did not seem to be mediated through neural pathways. Nor did the pressor response *per se* seem necessary. *L*-epinephrine in doses too small to change the arterial pressure or pulse rate evoked the typical renal functional alterations in two subjects. Other pressor agents (angiotonin, S-methyl isothiourea sulfate, and ephedrine) failed to produce exactly the same response. Sodium and potassium output were lowered by angiotonin (three subjects) and S-methyl isothiourea (two subjects) but the glomerular filtration rate and urine flow were also decreased in association with the reduction in renal blood flow. Ephedrine (four subjects) induced renal vasoconstriction in proportion to the elevation in blood pressure so that renal blood flow did not change. The potassium excretion decreased but sodium output rose without change in filtration.

Sodium reabsorption always seemed to increase in association with a fall in renal blood flow. When renal blood flow remained unchanged as in two of the subjects receiving *l*-norepinephrine and in the four subjects receiving ephedrine, the

pressor response was associated with diminished tubular reabsorption of sodium. Potassium retention, on the other hand, appeared to be correlated only with intrarenal vasoconstriction. It is impossible to decide on the basis of these findings whether *l*-epinephrine and *l*-norepinephrine act directly upon the renal tubular cells, but the data suggest an indirect action mediated through local hemodynamic adjustments.

ACKNOWLEDGMENT

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REFERENCES

1. Nickel, J. F., Smythe, C. McC., and Bradley, S. E., Effect of pressor agents on renal water and electrolyte excretion in man. Federation Proc., 1952, **11**, 113.
2. Goldenberg, M., Pines, K. L., Baldwin, E. deF., Greene, D. G., and Roh, C. E., The hemodynamic response of man to nor-epinephrine and epinephrine and its relation to the problem of hypertension. Am. J. Med., 1948, **5**, 792.
3. Barcroft, H., and Konzett, H., On the actions of noradrenaline, adrenaline, and isopropyl noradrenaline on the arterial blood pressure, heart rate, and muscle blood flow in man. J. Physiol., 1949, **110**, 194.
4. Chasis, H., Ranges, H. A., Goldring, W., and Smith, H. W., The control of renal blood flow and glomerular filtration in normal man. J. Clin. Invest., 1938, **17**, 683.
5. Ranges, H. A., and Bradley, S. E., Systemic and renal circulatory changes following the administration of adrenin, ephedrine and paredrinol to normal man. J. Clin. Invest., 1943, *22*, 687.
6. Barnett, A. J., Blacket, R. B., Depoorter, A. E., Sanderson, P. H., and Wilson, G. M., The action of noradrenaline in man and its relation to phæchromocytoma and hypertension. Clin. Sc., 1950, **9**, 151.
7. Smythe, C. McC., Nickel, J. F., and Bradley, S. E., The effect of epinephrine (USP), *l*-epinephrine, and *l*-norepinephrine on glomerular filtration rate, renal plasma flow, and the urinary excretion of sodium, potassium, and water in normal man. J. Clin. Invest., 1952, **31**, 499.
8. Jacobson, W. E., Hammarsten, J. F., and Heller, B. I., The effects of adrenaline upon renal function and electrolyte excretion. J. Clin. Invest., 1951, **30**, 1503.
9. Smith, H. W., Goldring, W., Chasis, H., Ranges, H. A., and Bradley, S. E., The William Henry Welch Lectures. II. The application of saturation methods to the study of glomerular and tubular function in the human kidney. J. Mt. Sinai Hosp., 1943, **10**, 59.
10. Baldwin, D. S., Schreiner, G. E., Breed, E. S., Wesson, L. G., Jr., and Maxwell, M. H., Depression of apparent p-aminohippurate extraction ratio by glucose. J. Clin. Invest., 1950, **29**, 614.
11. Goldring, W., and Chasis, H., Hypertension and Hypertensive Disease. New York, The Commonwealth Fund, 1944.
12. Bradley, S. E., Bradley, G. P., Tyson, C. J., Curry, J. J., and Blake, W. D., Renal function in renal diseases. Am. J. Med., 1950, **9**, 766.
13. Murphy, R. J. F., and Stead, E. A., Jr., Effects of exogenous and endogenous posterior pituitary antidiuretic hormone on water and electrolyte excretion. J. Clin. Invest., 1951, **30**, 1055.
14. Smith, H. W., Rovenstine, E. A., Goldring, W., Chasis, H., and Ranges, H. A., The effects of spinal anesthesia on the circulation in normal, unoperated man with reference to the autonomy of the arterioles, and especially those of the renal circulation. J. Clin. Invest., 1939, **18**, 319.
15. Bradley, S. E., and Parker, B., The hemodynamic effects of angiotonin in normal man. J. Clin. Invest., 1941, **20**, 715.
16. McGeorge, M., Sherif, M., and Smirk, F. H., Observations on the properties of S-methyl iso-thiourea sulphate with particular reference to the circulatory effects. J. Physiol., 1942, **100**, 474.
17. Maxwell, M. H., Morales, P., and Crowder, C. H., Jr., Effect of therapeutic doses of ephedrine on renal clearances in normal man. Proc. Soc. Exper. Biol. & Med., 1951, **77**, 539.
18. Smith, H. W., The Kidney: Structure and Function in Health and Disease. New York, Oxford Univ. Press, 1951.
19. Verney, E. B., Croonian lectures. Antidiuretic hormone and factors which determine its release. Proc. Roy. Soc., London, 1947, **B135**, 25.
20. Gaunt, R., Birnie, J. H., and Eversole, W. J., Adrenal cortex and water metabolism. Physiol. Rev., 1949, **29**, 281.
21. Pullman, T. N., and McClure, W. W., The effect of l-nor-adrenaline on electrolyte excretion in normal man. J. Lab. & Clin. Med., 1952, **39**, 711.
22. Griffith, F. R., Jr., Fact and theory regarding the calorigenic action of adrenalin. Physiol. Rev., 1951, **31**, 151.
23. Gaddum, J. H., The alkaloid ephedrine. Brit. Med. J., 1938, **1**, 713.

POSITIVE DIRECT COOMBS TEST INDUCED BY PHENYLHYDRAZINE[1]

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The Coombs or antiglobulin test was introduced for the detection of antibodies to erythrocytes of a type termed "incomplete antibodies" (1). The indirect Coombs test has gained wide application in the detection of the antibodies to various erythrocytic antigens (2) and in the cross-matching of blood for transfusions (3). The direct Coombs test has become a diagnostic and investigative tool in hemolytic disease of the newborn (4–6), following incompatible blood transfusions (7) and in the acquired hemolytic anemias, idiopathic or secondary types (8–22).

All of these conditions in man when associated with positive direct and indirect Coombs tests, have been interpreted to be the result of immune mechanisms, either of the isoimmune or autoimmune type. In the present communication the production of a positive direct Coombs test in the dog by means of the drug phenylhydrazine is described. The phenomenon is unattended by the immunologic implications of blood incompatibility and is not associated with the usual conditions causing a secondary or symptomatic acquired hemolytic anemia.

MATERIAL AND METHODS

Adult normal mongrel dogs of either sex weighing 5.5 to 11 kg. were used. After the control observations had been completed, a 1 to 2 per cent solution of phenylhydrazine hydrochloride (Merck) in saline was injected intravenously in a single dose of 40 mg. per kg. body weight. The studies were conducted periodically thereafter up to 41 days.

The hemoglobin concentration of the peripheral blood was determined by the alkaline hematin method (23). The hematocrit was determined according to Wintrobe (24) and the reading was corrected for trapped plasma according to the method of Chaplin and Mollison (25). Reticulocytes were estimated routinely by counting 1000 erythrocytes stained with brilliant cresyl blue (26). Heinz bodies were stained with 0.2 per cent methyl violet in 95 per cent ethyl alcohol and the percentage concentration of erythrocytes containing these structures was obtained by counting 1000 erythrocytes (27). The mechanical fragility of erythrocytes was determined by a modification of the method of Shen, Castle, and Fleming (28). One cc. of oxalated blood (5 cc. blood plus 10 mg. of a buffered mixture of potassium oxalate and ammonium oxalate) was placed in a 25 cc. Erlenmeyer flask containing 20 glass beads, each of about 4 mm. diameter. The flask was clamped to a plastic disc 16.5 cm. from the axis and rotated for 90 minutes at 30 rpm. at room temperature. The amount of hemoglobin in the supernatant plasma was related to the total hemoglobin concentration of the blood as per cent hemolysis.

The plasma hemoglobin concentration was determined by the method of Bing and Baker (29). The serum bilirubin concentration was determined by the method of Malloy and Evelyn (30).

The Coombs tests: The canine serum anti-serum was prepared in the rabbit by slight modifications of the directions of Mourant (31): Rabbits weighing 2 to 3 kg. were injected with pooled canine serum obtained by mixing about 5 cc. of serum from each of three to five dogs. A course of intravenous injections was given at 2-to-3-day intervals beginning with a 0.5 cc. dose and following with 5 doses of 1 cc. each. The animal was bled 10 days after the last dose. Pooled erythrocytes from normal dogs were used in absorbing the rabbit serum. The serum was then passed through a Seitz filter and aliquots of 1 cc. were stored in small sterile bottles at −20°C. until used. The results with this serum were compared on nine occasions with the results using a canine antiglobulin serum kindly furnished by Drs. L. E. Young and S. N. Swisher of Rochester, New York. When these two sera were used undiluted with cells giving positive results, the degree of agglutination evoked by the two was identical.

In the direct Coombs test one drop of a 2 per cent suspension of the canine erythrocytes was washed three times in abundant saline at room temperature. After the third wash the test tube was turned up and allowed to drain. To the erythrocytes suspended in the residual saline, a suspension approximating the original 2 per cent concentration, 2 drops of the canine serum anti-serum (Coombs serum) were added. The mixture was allowed to stand for 30 minutes following which it was centrifuged for one minute at 500 to 1000 rpm. After shaking the button of cells into the supernatant the results were evaluated grossly as follows: a solid button

[1] Supported by a grant from the U. S. Public Health Service, National Heart Institute.

of agglutinated cells was designated 4+, a few large clumps 3+, multiple scattered smaller clumps 2+, a definitely granular suspension with fine clumps 1+. All of these grossly detectable degrees of agglutination were checked by microscopic inspection which also demonstrated that the clumps of erythrocytes were stable for periods longer than 5 minutes. A smooth or fairly smooth suspension which upon microscopic inspection yielded definite but scattered clumps was designated ±. Rare microscopic clumps in a grossly negative preparation were ignored and the test was called negative. Since the ± designation is questionable, no significance is given to it in this presentation. The microscopic appearances are depicted in Figure 1.

The indirect Coombs test was conducted by incubating erythrocytes from normal dogs as a 2 per cent suspension at 37° C. for 30 minutes in the serum of the dogs receiving phenylhydrazine and then treating the cells as in the direct procedure. A random panel of canine erythrocytes obtained from three normal dogs was used throughout any one experiment.

The titration of the Coombs serum, with minimal modification, was conducted in accordance with the suggestion of Evans and Duane (11). Serial dilutions of the canine serum anti-serum with saline were prepared. To 0.1 cc. of the diluted serum was added 0.05 cc. of a 2 per cent suspension of washed erythrocytes. The mixture stood at room temperature for 30 minutes, then was

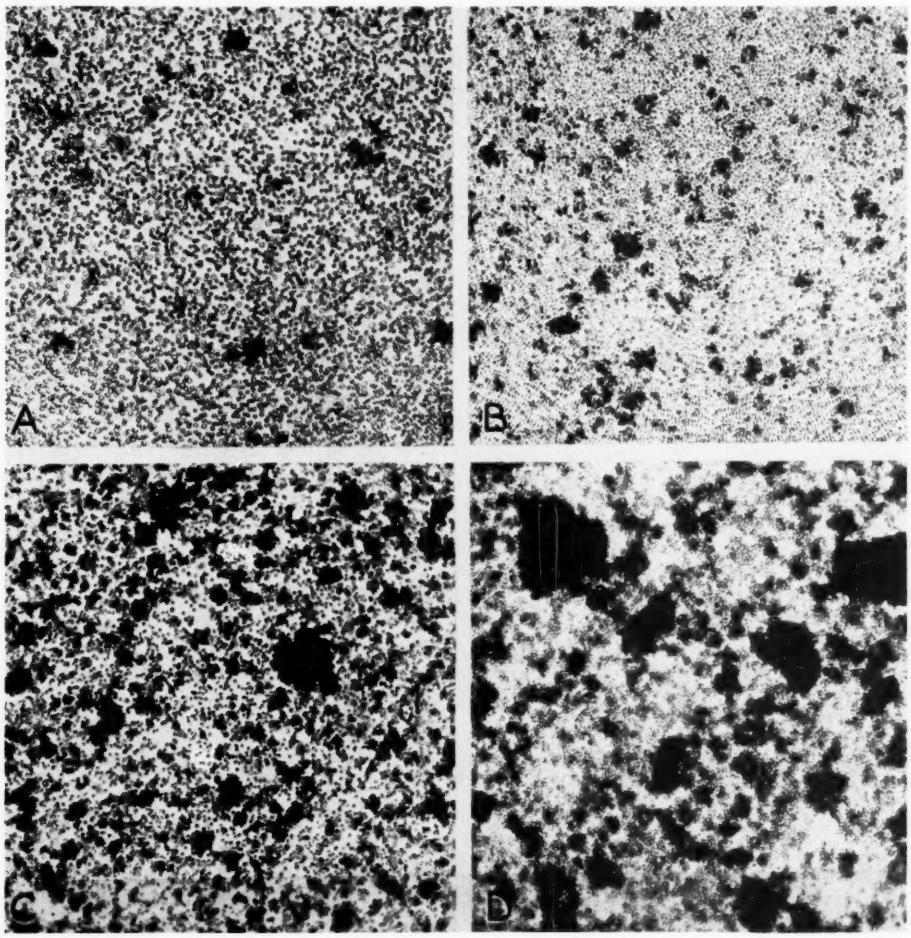

FIG. 1. MICROSCOPIC APPEARANCE OF THE VARIOUS GRADES OF AGGLUTINATION INDUCED BY THE DIRECT COOMBS TEST FOLLOWING PHENYLHYDRAZINE. (A) DEPICTS THE MICROSCOPIC APPEARANCE OF A GROSS ± REACTION; (B) A 1+ REACTION; (C) A 2+ REACTION; AND (D) A 3+ REACTION

centrifuged at 500 to 1000 rpm. for one minute and evaluated for clumping.

The trypsin-treated erythrocytes were prepared by a modification (32) of the description of Morton and Pickle (33). A 2 per cent solution of trypsin (Difco 1:250) in buffered saline (pH 7.3) was stored at −20° C. in small aliquots until used. A 1:10 dilution of this 2 per cent solution in buffered saline was used as a working solution. To 4.5 ml. of a 10 per cent suspension of washed RBC was added 0.5 ml. of the diluted trypsin solution. This mixture was incubated for 10 minutes at 37° C., centrifuged at 2500 rpm. for 10 minutes and the supernatant decanted. The cells were re-suspended to 2 per cent suspension in the buffered saline before using.

The test for "incomplete antibodies" using a colloid medium (34) was performed by the use of a final suspension of erythrocytes in 22 per cent human albumin solution according to the following steps: The 22 per cent solution was made up from a 25 per cent solution of human albumin in buffered diluent (E. R. Squibb and Sons, New York) by adding buffered saline (pH 7.3 to 7.5). To 0.1 cc. of 22 per cent albumin in a 13 × 75 mm. test tube was added 1 drop of a 2 per cent suspension of the test cells which had been washed three times in abundant saline. This was incubated at 37° C. for 30 minutes, centrifuged at 500 to 1000 rpm. for 1 minute and examined macroscopically for clumping. If no gross agglutination was evident the contents of the tube were poured on a slide and examined microscopically.

The eluates from the Coombs positive erythrocytes were prepared by an adaption of the Landsteiner-Miller procedure (35) as follows: 15 to 20 cc. of defibrinated blood were centrifuged, the serum was removed, the erythrocytes were washed four times with abundant saline, a final 50 per cent suspension of erythrocytes in saline was heated to 56° C. with constant agitation for 6 to 8 minutes. The saline was then separated while warm. As a result of this procedure hemolysis was mild to moderate. The ability of such eluates to produce direct agglutination and agglutination by means of the indirect Coombs procedure was tested by means of a panel of erythrocytes obtained from normal dogs. No direct agglutination was noted.

Since phenylhydrazine produces intravascular hemolysis and hemoglobinemia control tests were conducted in an attempt to determine whether hemolyzed canine blood *per se* could cause the erythrocytes obtained from normal dogs to agglutinate under the influence of the canine antiserum produced in the rabbit (canine Coombs serum) and used in the experiments here reported. The normal dogs used in these tests were not used for the injection of phenylhydrazine.

For this purpose the indirect Coombs test was conducted on the serum and hemolyzed erythrocytes obtained from two normal dogs. The serum and hemolyzed erythrocytes were incubated with the erythrocytes obtained from three additional normal dogs and the erythrocytes from the dog contributing the serum and hemolyzed erythrocytes. The incubation and the remainder of the indirect Coombs test was conducted as described above. The hemolyzed erythrocytes were prepared as follows: the erythrocytes from the normal animals used were washed three times with abundant saline, a final suspension of erythrocytes in saline was prepared with the same concentration of erythrocytes as originally present in the blood, this suspension was hemolyzed by repeated freezing and thawing. Altogether there were eight tests against the sera and eight tests against the hemolyzed fractions. In all instances the indirect Coombs test was negative.

The influence of hemolysis *per se* on the Coombs test was appraised also by the injection of 300 cc. of hemolyzed blood, obtained by repeated freezing and thawing, into a previously unused normal dog. The effect of this procedure on the direct Coombs test, Heinz body concentration and mechanical fragility of the recipient's circulating erythrocytes was noted periodically for one week. These tests yielded negative results.

These observations indicated that hemolysis *per se* did not cause a false positive Coombs test with the canine antiserum prepared in this laboratory.

RESULTS

Direct Coombs test

a) Results with the undiluted Coombs serum (Table I). The results from seven dogs followed up to 41 days after the injection of phenylhydrazine are summarized in Table I. The direct Coombs test became positive within two to three days. The gross agglutination varied between 1 and 3 +. The test was observed in these dogs to remain positive for 16 days. It became negative between 16 and 28 days. Dogs Nos. 6 and 7 (Table I) received additional doses of phenylhydrazine after the positive direct Coombs test had disappeared. The cycle of positive direct Coombs test repeated itself in a manner similar to that observed following the first injection.

b) Results with serial dilutions of Coombs serum with saline; the titer of Coombs serum; the prozone phenomenon (Tables II, III, and IV). Serial dilutions of the canine-serum anti-serum (Coombs serum from the rabbit) were prepared with saline. The diluted serum was added to washed 2 per cent suspensions of the canine erythrocytes after the injection of phenylhydrazine. The procedure was conducted periodically with the red cells from dogs Nos. 1, 2, 3, 7, 8, 9, 10, and 11 as shown in Tables II, III, and IV. The dilutions of 1:8 to 1:512 yielded positive results at 3 to 6 days. Thereafter the titer gradually receded except for dog No. 3, which expired and revealed a terminal elevation of the titer.

Absent or weaker agglutinations at lower dilutions followed by stronger agglutinations at higher dilutions were noted in 8 of 32 titrations on the 8 dogs tested. This phenomenon had the characteristics of a prozone (36) on four occasions in the sense of a gradual elevation in the intensity of agglutination as succeeding higher dilutions of the serum were used. In the other four examples the phenomenon was prozone-like in that the change was sudden from no agglutination to a positive result with higher dilutions. This blocking out of the positive test with the undiluted serum possibly explains the negative results noted with dogs Nos. 3, 5, and 7 on days 6, 13, and 5, respectively.

TABLE I

Phenylhydrazine (40 mg. per kg.) was given following the observations on zero day *†

Dog No. Wt. *kg.*	Day	Direct Coombs test	Hb. *gm./100 cc.*	Hematocrit %	Heinz body conc. %	Mech. fragility % *Hem.*	Reticulocytes %
	0	Neg.	11.0	36.4	0	4.4	0.3
	1	Neg.	9.4	31.3	58.0	29.5	0.7
	3	1+	6.1	20.7	82.2	31.1	1.8
	6	1+	2.2	14.0	54.0	25.3	11.4
	10	1+	6.0	24.5	7.6	28.2	7.6
(1)	14	1+	7.3	29.8	0.2	56.7	5.7
	16	1+	7.1	27.5	0.1	43.9	1.4
11	20	±	8.7	29.7	0	45.0	0.7
	24	±	7.6	31.4	0	15.2	0.6
	28	Neg.	8.9	29.4	0	67.3	
	30	±	10.8	34.5	0.1	33.9	0.1
	35	Neg.	9.2	31.2	0	57.4	0.3
	41	Neg.	11.8	32.4	0	17.0	0.5
	0	Neg.	14.0	43.3	0	7.0	0.6
	1	±	10.2	33.3	60.0	13.0	1.8
	3	1+	7.9	24.6	75.7	47.7	4.4
	6	1+	6.6	26.1	41.5	28.7	9.9
(2)	10	±	9.4	30.9	30.0	54.2	2.6
	14	±	9.8	37.2	1.5	58.3	2.9
6.4	17	±	10.6	36.3	0	35.2	1.1
	20	±	12.0	39.2	0	6.2	0.5
	24	Neg.	10.6	37.2	0	40.6	0.3
	28	Neg.	10.8	39.1	0	81.6	
	30	±	12.5	40.4	0.1	24.8	0.1
	35	Neg.	12.8	37.2	0.9	9.2	0.1
	41	Neg.	13.5	43.5	0	40.0	0.4
	0	Neg.	14.4	44.9	0	7.3	0.1
	1	Neg.	10.0	37.2	72.0	12.5	0.4
	3	1+	7.2	23.0	85.6	73.4	1.3
(3)	6	Neg.	4.7	19.7	48.0	50.0	14.6
	10	1+	5.2	20.6	16.1	97.0	3.4
	14	±	5.2	22.8	0.3	21.3	1.9
5.5	17	3+	6.0	23.1	0.2	40.8	2.9
	Expired						
	2	1+	14.0		47.0		
	3	1+	7.9	26.0	60.0		
	6	3+	3.5	16.0	64.0		
	7	3+			52.0		
(4)	12	2+			9.0		
	13	1+			5.8		
11	15	1+	6.4	27.2	4.7		
	16	Neg.			1.6		
	19	Neg.	7.5	29.9	1.4		
	21	Neg.	8.0				
	28	Neg.			0.7		

* The direct Coombs test was obtained by the use of the undiluted canine serum anti-serum (Coombs serum).
† Hb: Hemoglobin concentration.
Heinz body concentration: Concentration Heinz body % of 1,000 RBC.
Mechanical fragility: Mechanical fragility RBC % hemoglobin liberated.

TABLE I—*Continued*

Dog No. Wt. kg.	Day	Direct Coombs test	Hb. gm./100 cc.	Hematocrit %	Heinz body conc. %	Mech. fragility % Hem.	Reticulocytes %
	2	1+	11.5	27.5	54.0		
	3	1+	8.4	23.0	61.0		
	6	2+	5.1	20.0	47.0		
	8	3+			39.0		
(5)	12	1+		30.0	5.0		
	13	Neg.		28.0	3.7		
6.4	15	3+	7.6	27.8	1.8		
	16	3+		30.2	0.7		
	19	1+	8.4	30.4	0.4		
	21	3+	9.1	30.0			
	28	±			0.3		
	44‡	Neg.	13.1	41.0	0	5.9	
	45	±	11.6	40.9	74.0	22.0	
	48	±	8.0	30.2	77.4	42.6	
	51	±	11.2	34.3	31.8	11.8	
(6)	56	±	10.6	33.9	6.7	37.8	
9.0	66	1+	11.0	34.0	0	27.0	
	69	±	10.6	34.1	0	76.4	
	85§	Neg.	10.6	34.1	0	5.6	
	87	±	7.1	36.4	83.6	33.8	
	91	1+	5.8	21.8	57.9	45.1	
	93	1+	7.6	25.5	23.6	10.3	
	98	±	8.4	30.4	2.7	55.5	
	0	Neg.	10.9	35.4	0	5.8	
	1	±	9.6	35.4	29.0	9.0	
	3	1+		37.0	52.0	25.0	
	5	Neg.	9.6	32.0	32.0	14.3	
	7	2+	7.1	26.3	16.0	3.2	
	10	1+	7.1	29.7	1.5	6.5	
	13	1+	10.2	35.6	0.2	8.2	
(7)	18	Neg.	10.2	35.6	0	10.2	
11.4	28	Neg.	11.8	38.3	0	5.4	
	47‡	Neg.	12.3	40.0	0		
	49	1+	7.1		92.2	27.3	
	50	2+	7.6			15.7	
	53	2+	4.4	19.3	56.5	7.8	
	55	2+	6.9	25.4	12.8	4.5	
	60	±	8.1	30.4	6.7	13.8	

‡ Second dose of phenylhydrazine 44 and 47 days after first dose.
§ Third dose phenylhydrazine 41 days after second dose.

as shown in Table I. These negative results with the undiluted serum were preceded and followed by positive results.

c) Potentiation of the positive direct Coombs test with trypsin (Table III). Subjecting the erythrocytes to the action of trypsin prior to the addition of the canine serum anti-serum accentuated the degree of agglutination. The treated cells also carried the test in the titration of the serum one to several tubes beyond that observed without the action of the enzyme as noted in eight comparisons conducted on dogs Nos. 1, 2, 7, and 9. Trypsin alone, under the conditions used, was not able to affect agglutination of the erythrocytes. Eventually, even with trypsin, the test became negative.

d) Neutralization of the Coombs serum from the rabbit with canine serum (Table IV). The Coombs serum was diluted serially with saline for one set of tests. For another set of tests on the same erythrocytes the Coombs serum was diluted serially in the same manner with canine serum prepared by pooling equal parts of serum obtained from four normal dogs. The Coombs serum and normal canine serum were allowed to interact for 30 minutes before use. The erythrocytes tested yielded a positive direct Coombs test following the injection of phenylhydrazine. This procedure was

TABLE II

Data on the Coombs titer obtained by serial dilution of the Coombs serum with saline and its addition to washed erythrocytes following the injection of phenylhydrazine

Dog No.	Day	Serial dilutions of Coombs serum 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
(1)	3	0	0	0	+	+	+	+	+				
	5	+	+	+	+	+	±	±	±	±	±	0	
	10	+	+	+	+	±	±	±	±	0	0	0	
	14	+	+	+	+	±	±	±	±	0	0	0	
	16	+	±	±	±	±	±	±	0	0	0		
	20	±	±	±	±	±	±	±	±	±	±	0	
	24	±	±	±	±	±	±	0	0	0	0	0	
	30	±	±	±	0	0	0	0	0	0	0	0	0
(2)	6	+	+	+	+	+	0	0	0	+	±	0	0
	14	±	±	±	±	0	0	0	0	0	0	0	
	17	±	±	±	0	0	0	0	0	0	0		
	20	±	±	0	0	0	0	0	0	0	0	0	
	24	0	0	0	0	0	0	0	0	0	0	0	
(3)	3	+	0	0	0	0	0	0	0	+	+	±	0
	6	0	0	0	0	0	±	±	±	±	±	±	0
	10	+	±	±	±	±	±	±	±	+	0	0	0
	14	±	±	±	+	++	+	+	+	±	±	±	0
	17	+++	+++	+++	+++	+++	++	++	++	±	0	0	0
	Expired												

conducted a total of seven times on the erythrocytes from four dogs (Dogs Nos. 8, 9, 10, and 11). The direct Coombs test was positive with the saline dilutions of the Coombs serum of 1:8 to 1:64. Dilution and incubation of the Coombs serum with the pooled canine serum neutralized the ability of the Coombs serum to cause agglutination of the erythrocytes in six of the seven tests conducted and minimized it on the seventh occasion. This type of neutralization suggests that antibodies in the Coombs serum reacted with corresponding canine proteins on the affected erythrocytes in causing the agglutination.

e) Results with the serum from normal rabbits (Table IV). The washed erythrocytes which yielded a positive direct Coombs test with the

TABLE III

Data on the Coombs titer obtained with untreated erythrocytes and with the same cells after treatment with trypsin

Dog	Day	Coombs method	Serial dilutions of Coombs serum 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
(1)	24	Regular	±	±	±	±	±	±	0	0	0	0	0	
		Trypsin	++	±	±	±	±	±	±	0	0	0	0	
	30	Regular	±	±	±	0	0	0	0	0	0	0	0	0
		Trypsin	±	±	±	±	0	0	0	0	0	0	0	0
(2)	24	Regular	0	0	0	0	0	0	0	0	0	0	0	
		Trypsin	+	±	0	0	+	+	0	0	0	0	0	
	30	Regular	±	0	0	0	0	0	0	0	0	0	0	0
		Trypsin	±	±	±	0	0	0	0	0	0	0	0	0
(7)	2	Regular	0	±	±	±	±	±	0	0	0	0	0	
		Trypsin		+	+	+	+	±	±	±	0	0	0	
	6	Regular	+	+	+	+	±	±	±	0	0	0	0	0
		Trypsin	+	+	+	+	+	+	±	±	±	0	0	0
(9)	2	Regular	+	+	+	+	+	++	+	±	±	±	0	
		Trypsin	+	+	++	++	++	++	+	±	±	±	±	
	8	Regular	++	++	++	++	+	+	±	±	0	0	0	0
		Trypsin	++	++	++	++	++	++	+	+	+	0	0	0

TABLE IV

The neutralization of the Coombs serum by means of canine serum is demonstrated. Failure of direct Coombs positive erythrocytes to agglutinate in normal rabbit serum, normal canine serum and concentrated albumin is also depicted

Dog No.	Day	Procedure	Serial dilutions of Coombs serum 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	RBC + Dog serum	RBC + Rab. ser.	RBC + Conc. alb.
8	5	Coombs serum + Saline dilution	1+	1+	1+	1+	+	+	+	0	0	0	0	0	0	0	0
		Coombs serum + Dog serum dil.		±	0	0	0	0	0	0	0	0	0	0			
	7	Coombs serum + Dog serum dil.		±	±	0	0	0	0	0	0	0	0	0			
9	4	Coombs serum + Saline dilution	1+	1+	1+	1+	±	0	0	0	0	0	0	0	0		0
		Coombs serum + Dog serum dil.		2+	±	±	±	±	±	0	0	0	0	0			
10	3	Coombs serum + Saline dilution	1+	1+	1+	±	±	0	0	0	0	0	0	0	0		0
		Coombs serum + Dog serum dil.	0	0	0	0											
	4	Coombs serum + Saline dilution	±	±	±	0	0	0	0	0	0	0	0	0	0	0	0
		Coombs serum + Dog serum dil.	0	0	0	0	0	0	0	0	0	0	0	0			
11	3	Coombs serum + Saline dilution	±	±	1+	2+	1+	2+	1+	±	±	0	0	0	0		0
		Coombs serum + Dog serum dil.				0											
	4	Coombs serum + Saline dilution	1+	1+	2+	2+	1+	1+	±	0	0	0	0		0	0	0
		Coombs serum + Dog serum dil.		0	0	0	0	0	0	0	0	0	0				

saline dilutions of the Coombs serum up to 1:8 to 1:64 failed to agglutinate when placed in the serum from normal rabbits. The same washed erythrocytes also failed to agglutinate in the serum from a normal dog and in a concentrated human albumin solution.

TABLE V

Results of the indirect Coombs test utilizing a panel of 3 or 4 erythrocytes from normal dogs and the serum and eluate from the blood at the time the direct Coombs test was prominently positive

Dog No.	Day	Direct Coombs	Indirect Coombs with	Panel RBC 1	2	3	4
(3)	17	3+	Serum	±	±	1+	
			Eluate	±	±	1+	
(4)	7	3+	Serum	0	0	0	0
			Eluate	0	0	0	±
(5)	7	3+	Serum	0		0	0
			Eluate	±		±	±
(9)*	10	2+	Serum	1+	1+	±	
			Eluate	±	1+	1+	

* Second dose.

The albumin test

It has been demonstrated that "incomplete antibodies" coating erythrocytes can also be detected by agglutination within a colloid medium, such as 22 per cent serum albumin. The albumin test following phenylhydrazine was consistently negative while the direct Coombs test was positive. This observation was made repeatedly with the erythrocytes of eight dogs (Nos. 1, 2, 3, 7, 8, 9, 10, and 11).

Elution of direct Coombs positive erythrocytes (Tables V and VI)

Eluates were prepared by the Landsteiner-Miller procedure from the erythrocytes of six

TABLE VI

The Coombs titer as obtained by serial dilution of the Coombs serum with saline and the addition of this serum to the erythrocytes of three normal dogs after they had been incubated with the serum and erythrocytic eluate both obtained at the time that the direct Coombs test was positive

RBC No.	Serum (S) or Eluate (E)	Serial dilutions of Coombs serum with saline											
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
1	S	+	+	+	+	±	±	±	±	±	±	0	0
	E	±	±	+	+	+	+	+	+	±	±	±	0
2	S	+	+	±	±	±	±	±	±	±	±	0	0
	E	+	+	+	+	+	+	±	±	±	±	0	0
3	S	±	±	±	±	±	±	±	0	0	0	0	0
	E	+	+	+	+	±	±	±	±	0	0	0	0

dogs (Nos. 1, 2, 3, 4, 5, and 9) at the time the direct Coombs test was positive with the undiluted Coombs serum. The eluate and the serum obtained at the same time were tested by means of the indirect Coombs test against the erythrocytes obtained from three or four normal dogs. In Table V the indirect Coombs test was conducted with undiluted Coombs serum while in Table VI serial dilutions of Coombs serum with saline were used in the indirect Coombs test as applied to the serum and eluate of one dog (No. 9). The eluates of two of the four dogs tested yielded positive indirect Coombs test. The eluates and the corresponding sera gave similar results. These observations indicate that the causative agent of the positive direct Coombs test following phenylhydrazine is elutable but does not elucidate the nature of the eluted material.

The indirect Coombs test (Table VII)

The indirect Coombs test was applied to the serum of the dogs receiving phenylhydrazine utilizing the same panel of erythrocytes from three normal dogs throughout each experiment. It should be noted in Table VII that the indirect Coombs test was negative in all but one case prior to the injection of the drug but that it subsequently became slightly positive in all cases. Although these observations have not been elucidated, they merit some comment.

The development of the positive indirect Coombs test to certain red cells cannot be ascribed to isoimmunization since no blood transfusions were involved. Three alternative explanations for this phenomenon may be considered: 1) That the drug or its hemolytic effects potentiated or unmasked the presence of low titered natural antibodies against certain red cells from other animals; 2) that the material in the serum causing the positive indirect Coombs test was derived from the erythrocytes altered by the drug and yielding a positive direct Coombs test, just as a positive test was obtained by elution; and 3) that the drug altered normal proteins in the plasma, thus making these adsorb onto erythrocytes.

Relation of general hematologic features following phenylhydrazine to the positive direct Coombs test (Table I)

a) Relation to the anemia. It is apparent in Table I that the magnitude of the positive direct

TABLE VII

Data on the indirect Coombs test utilizing the serum following phenylhydrazine and a panel of erythrocytes obtained from three normal dogs

Dog	Day	Indirect Coombs test panel cells		
		1	2	3
(1)	0	0	0	+
	4	±	±	+
	7	±	0	Hem.
	11	+	±	+
	15	+	+	+
	17	0	0	±
	21	+	±	+
	25	±	±	±
(2)	0	0	0	0
	4	±	±	0
	7	0	0	0
	15	±	+	±
	18	+	±	±
	21	±	±	+
	25	+	±	±
(3)	0	0	±	0
	4	+	+	+
	7	0	0	0
	11	+	+	++
	15	+	+	+
	18	±	±	+

Coombs test bore no constant relationship to the degree of anemia induced by the phenylhydrazine. Thus the positive test not only occurred at height of the anemia, as demonstrated by the hemoglobin concentration and the hematocrit reading, but persisted and at times became more prominent during recovery from the anemia.

b) Relation to Heinz bodies. In these studies the appearance and disappearance of the Heinz bodies within the erythrocytes, as expressed by the percentage of erythrocytes containing these structures, resembled that described by Cruz (5). Thus the Heinz body concentration reached a maximum in 3 to 6 days, and receded to the base line in 13 to 18 days. There was no constant relationship between the proportion of erythrocytes containing Heinz bodies and the reactivity of the erythrocytes with Coombs serum. The direct Coombs test remained positive after the concentration of Heinz bodies receded to or near extinction. This point was emphasized further in two dogs in which the Heinz body concentration was determined at 1.5, 3, 4.5, and 24 hours following the injection of the drug. The concentration of Heinz bodies at 4.5 hours was 28 per cent for both dogs and at 24 hours the concentrations were 37 and 53 per cent. Yet during the entire interval, the direct Coombs test remained negative, even when serial dilutions of the Coombs serum with saline were used to avoid the prozone phenomenon.

The Heinz body has been interpreted to result from alteration in the structure of the red cell (38, 39). According to Ponder (39) the structure consists mainly of denatured globin. Since it appears to possess a protein component the possibility that the altered protein structure might attract the canine serum anti-serum of the rabbit required consideration. These observations indicate that the Heinz bodies *per se* were not the structures causing the positive direct Coombs test.

c) Relation to mechanical fragility of erythrocytes. Following the injection of phenylhydrazine the mechanical fragility of fresh erythrocytes was prominently elevated (Table I) from an average control value of 5.8 per cent hemolysis (4.4 to 7.0 per cent) to values varying between 25 and 97 per cent, usually 57 to 77 per cent. The mechanical fragility reached a maximum at 11 to 15 days in four of five dogs so studied, at which time the direct Coombs test was positive. This degree of mechanical fragility then receded as the direct Coombs test tended to become negative, but in all dogs so studied (Nos. 1, 2, 3, 7, and 9) the fragility reached additional maxima and at times (dogs Nos. 1, 2, 7, and 9) when the direct Coombs test became negative.

d) Relation to reticulocytes. The reticulocyte count (dogs 1, 2, and 3) did not become prominently elevated until the anemia reached its maximum. There seemed to be a lag of 3 to 6 days before the upsurge of reticulocytes, despite the prominent stimulus of hemolysis. The reticulocytosis receded at about 15 days. Thus the reticulocytosis was prominent during the interval when the direct Coombs test was positive; however, at times the Coombs test remained positive or increased in intensity (dog No. 3) after the reticulocytosis had receded.

e) Relation to hemoglobinemia and jaundice. The positive direct Coombs test was questionable or positive at the height of the hemoglobinemia induced by phenylhydrazine but remained the same or became more positive after the recession of the elevated concentration of plasma hemoglobin. Jaundice was not prominent in these experiments and as a consequence appeared unrelated to the positive direct Coombs test.

Direct Coombs test following addition of phenylhydrazine to blood in the test tube

Phenylhydrazine produces hemolysis and Heinz bodies within the erythrocytes when the drug is added to blood in the test tube, thus demonstrating that the drug injures red cells *in vitro*. This finding suggested the need to test the direct Coombs test following action of the drug in the test tube. Accordingly, to 1 cc. aliquots of oxalated canine blood was added 0.5, 0.25, 0.1, and 0.05 mg. of phenylhydrazine. The first three tubes were allowed to stand at room temperature for 0.25, 1.0, 2.5 to 3.0 hours, respectively, and the last tube was kept in a 37° C. water bath for 18 to 24 hours. At the end of these intervals the erythrocytes were checked for the presence of Heinz bodies and were washed and subjected to the direct Coombs test.

Blood obtained from six normal dogs was so treated. All preparations revealed the presence

of Heinz bodies. In the gross appraisal of the direct Coombs test the erythrocytes in 10 of the 23 tests appeared slightly to moderately clumped (1 + to 2 + results) but upon microscopic inspection the clumps disintegrated after 1 to 3 minutes. The aggregates, which corroborated the gross appearance, appeared to be true clumps and did not resemble coarse rouleaux or so-called red cell "drifts" (4). This transient agglutination suggests a tendency following treatment in the test tube toward the same phenomenon observed after the intravenous injection of the drug. Further observations are required to clarify this possibility. A distinct difference, however, was noted between the agglutination in the direct Coombs test following the injection of phenylhydrazine into dogs, for in these instances the agglutination remained stable for the period observed, which always exceeded 5 minutes and in some special instances exceeded one hour.

DISCUSSION

It is commonly assumed that erythrocytes yielding a positive direct Coombs test are coated with antibodies of the "incomplete type" (1–20). This interpretation, dependent on an antibody response to an antigen, appears definite in certain conditions, such as hemolytic disease of the newborn (4–6) and following certain incompatible blood transfusions (7). In other states the antibody interpretation for a positive direct Coombs test is attractive although not as well founded. Thus, in the acquired hemolytic anemias the positive direct Coombs test has been considered to be a consequence of autoimmune mechanisms (8–17, 40). This concept has gained considerable support by the demonstration of specificities of certain of these "antibodies" in acquired hemolytic anemia (41, 42). Attractive as this hypothesis is the possibility of the production of abnormal globulins capable of attachment to erythrocytes by non-antigenic pathways cannot be ignored (21, 22).

While the literature on the appraisal of the direct Coombs test in man is extensive, few observations on its production in experimental animals have been cited in support of the antibody hypothesis. Noteworthy in this respect are the observations of Young and associates (43–45) on the production of a positive direct and indirect Coombs test in the dog by infusing incompatible blood and in canine hemolytic disease of the newborn in which "incomplete antibodies" were implicated as the causative factor. Chute and Sommers (46) observed a positive indirect Coombs test in parabiotic disease of the rat. The finding was interpreted as resulting from erythrocytic incompatibility causing "incomplete antibodies" in one parabiont which were exchanged through the common circulation and affected the erythrocytes of the partner.

Three important features of the pattern of development of the positive Coombs test in these experiments militate strongly against an antigen-antibody interpretation, and emphasize the weakness of this hypothesis as a general explanation for all instances of positive Coombs test. First, the rapid appearance of the positive Coombs test, within 2 days following injection of the drug, constitutes a strong objection to an antigen-antibody response. Second, the short-lived state of the positive test, as indicated by its disappearance in two to four weeks, is such as to be unusual for an antibody response. Finally, the lack of potentiation of the test by subsequent doses of the drug and the seemingly stereotyped response to subsequent doses do not connote immunologic characteristics.

The drug used, phenylhydrazine, is known to produce direct injury to erythrocytes which is associated not only with hemolysis but also with alterations within the erythrocytes, as demonstrated by the production of methemoglobin and Heinz bodies. The pattern of observations suggests that phenylhydrazine alters the red blood cells in such manner as to change their capacity to react with the canine serum anti-serum of the rabbit. Three theoretical possibilities for these alterations require further experimentation. In one the drug can be considered to alter the surface of the erythrocyte so as to make it act directly as a receptor for the canine antibodies. In another the drug can be considered to alter the surface of the erythrocyte so as to cause it to adsorb non-immune proteins, as normal plasma proteins. Finally, it is conceivable that the drug itself acts as the coupling agent between the surface of the erythrocyte and non-immune proteins, as plasma proteins.

While certain features of this study may be considered to constitute objections to an anti-

body response as the cause for the positive direct Coombs test, the findings of positive indirect Coombs test by the use of the serum and eluates from the erythrocytes have been cited in other circumstances as supporting an immune response. It is difficult to accept such an explanation from the present results, inasmuch as the pattern of development of the positive Coombs test in these experiments has, for reasons previously cited, features which cannot be ascribed to antigen-antibody reactions. An alternative hypothesis would ascribe the positive indirect Coombs test to the same general process of action by the drug which seems responsible for the positive direct Coombs test. Thus, according to this view, the factors causing the positive direct Coombs test might become detached from the erythrocytes, pass into the serum and be reabsorbed by other erythrocytes. It is not clear, as yet, what these factors are. They may be degradation products of injured erythrocytes or altered normal proteins.

These considerations, based on the observations available in the literature and the observations herein recorded, make possible a reappraisal of the mechanisms evoking a positive direct Coombs test. It seems definite that a positive direct Coombs test may result from established antigen-antibody reactions, as in hemolytic disease of the newborn and following certain incompatible blood transfusions. In the acquired hemolytic anemias, idiopathic and certain secondary types, the affected individual's erythrocytes are coated with globulins which resemble "incomplete antibodies." This has led to the interpretation that the globulins are autoimmune, although the presence of abnormal non-immune globulins capable of adsorption to erythrocytes has not been excluded. The present experiments indicate the production of the positive direct Coombs test by the action of a drug, as phenylhydrazine. The findings suggest an action of the drug on the erythrocytes as the cause for this phenomenon.

SUMMARY AND CONCLUSIONS

1. The drug phenylhydrazine has produced a positive direct Coombs test. The agglutination has been stable when the drug was injected intravenously into dogs and transient and unstable when the drug acted on the blood in the test tube.

2. The phenomenon does not appear to result from immunologic mechanisms for the reasons discussed.

3. The positive direct Coombs test appears to result from an alteration of the erythrocyte by phenylhydrazine or some breakdown product of phenylhydrazine.

ACKNOWLEDGMENT

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REFERENCES

1. Coombs, R. R. A., Mourant, A. E., and Race, R. R., A new test for the detection of weak and "incomplete" Rh agglutinins. Brit. J. Exper. Path., 1945, **26**, 255.
2. Race, R. R., and Sanger, R., Blood Groups in Man. Springfield, Ill., Charles C Thomas, 1950.
3. Muirhead, E. E., Problems in blood transfusion. III. Minor incompatible blood transfusions and acquired hemolytic anemia. Texas State J. Med., 1954, **50**, 465.
4. Coombs, R. R. A., Mourant, A. E., and Race, R. R., In-vivo isosensitisation of red cells in babies with hæmolytic disease. Lancet, 1946, **1**, 264.
5. Hill, J. M., and Haberman, S., Demonstration of Rh antibodies in the newborn and further evidence of the pathogenesis of erythroblastosis. J. Lab. & Clin. Med., 1946, **31**, 1053.
6. Levine, P., Recent developments in iso-immunization by the Rh factor. Am. J. Obst. & Gynec., 1945, **49**, 810.
7. Mollison, P. L., Blood Transfusion in Clinical Medicine. Springfield, Ill., Charles C Thomas, 1951, pp. 240, 286, 222.
8. Boorman, K. E., Dodd, B. E., and Loutit, J. F., Haemolytic icterus (acholuric jaundice) congenital and acquired. Lancet, 1946, **1**, 812.
9. Sturgeon, P., A new antibody in serum of patients with acquired hemolytic anemia. Science, 1947, **106**, 293.
10. Evans, R. S., Duane, R. T., and Behrendt, V., Demonstration of antibodies in acquired hemolytic anemia with anti-human globulin serum. Proc. Soc. Exper. Biol. & Med., 1947, **64**, 372.
11. Evans, R. S., and Duane, R. T., Acquired hemolytic anemia. I. The relation of erythrocyte antibody production to activity of the disease. II. The significance of thrombocytopenia and leucopenia. Blood, 1949, **4**, 1196.
12. Dacie, J. V., and de Gruchy, G. C., Auto-antibodies

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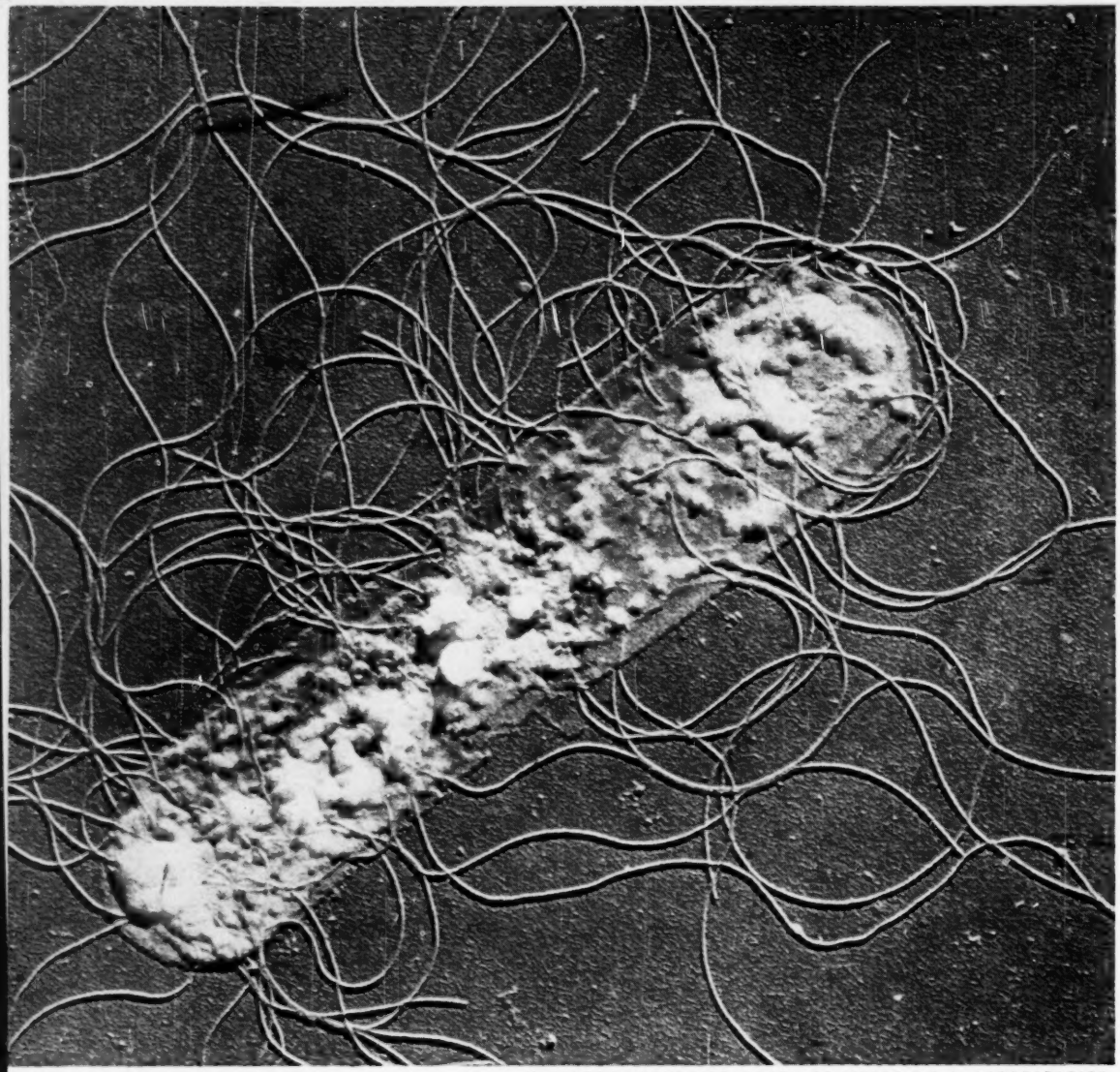

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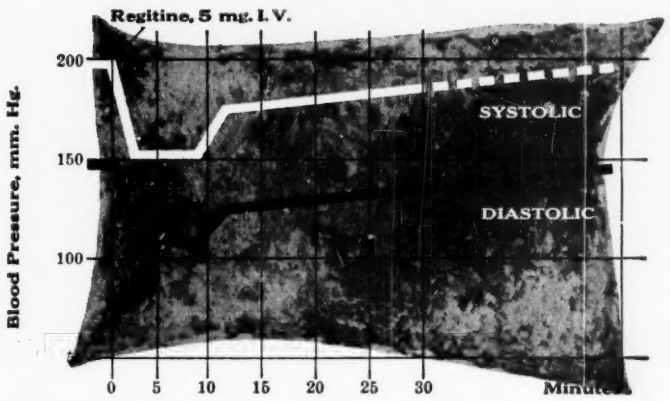

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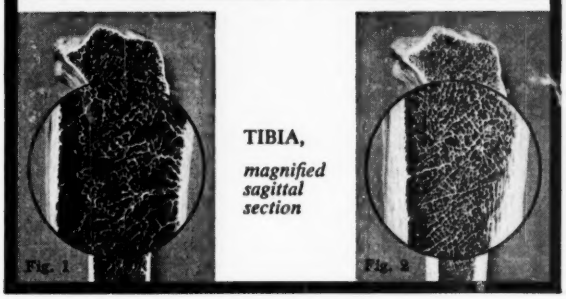

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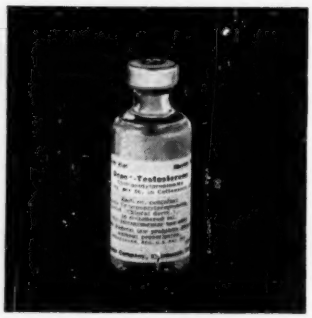

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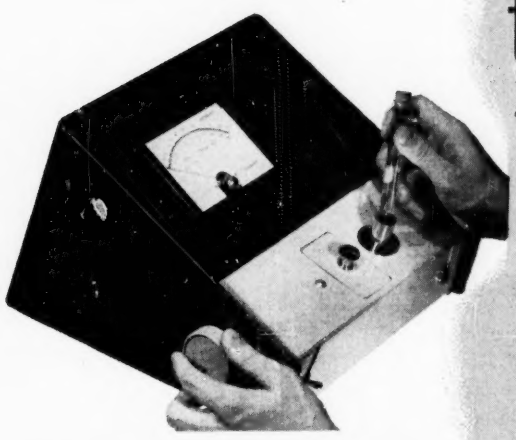

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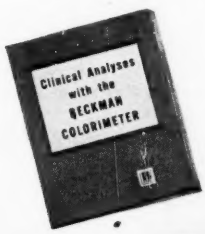

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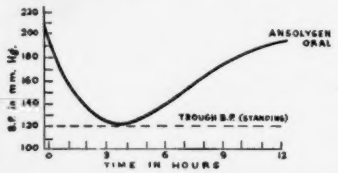

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1. Freis, E. D.: Personal communication
2. Sturgis, C. C.: Television Symposium; "The Management of Hypertension," American College of Physicians, Sept. 23, 1954
3. Smirk, F. H.: Lancet *1*:457 (March 7) 1953
4. Freis, E. D.: M. Ann. District of Columbia *23*:363 (July) 1954
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